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14. Abstract

Project #1

Background: Muscle injuries, especially pulls and strains, are among the most common and most frequently disabling injuries sustained by athletes and soldiers. Although injured muscles heal naturally, the regeneration is very slow and often yields incomplete functional recovery. In injured muscle, regeneration begins shortly after injury, but the healing process is rather inefficient and is hindered by fibrosis—that is, scar tissue formation. More importantly, the scar tissue that often replaces damaged myofibers may contribute to the tendency of strains to recur. We have observed that TGF-β1 plays a central role in skeletal muscle fibrosis and, more importantly, that the use of antifibrosis agents that inactivate this molecule, such as suramin (a Food and Drug Association [FDA]-approved drug that prevents fibrosis due to skin disorders), can reduce muscle fibrosis and consequently improve muscle healing, resulting in nearly complete recovery after laceration or strain injuries.

Objective/Hypothesis/Specific Aims/Study Design: We plan to develop biological approaches based on suramin to efficiently prevent the scarring process by blocking the action of TGF-β1; we will determine the appropriate time at which to administer suramin and the optimal suramin dosage after muscle contusion, a common military injury (**Technical Objective #1**). Because we have observed that suramin can both enhance muscle regeneration and neutralize the fibrotic effect of TGF-β1, we also propose experiments designed to further evaluate the beneficial effects of suramin on muscle regeneration (**Technical Objective #2A**). Finally, we will determine if this effect is mediated through suramin's interaction with muscle growth regulators, particularly its possible down-regulation of myostatin or up-regulation of follistatin (**Technical Objective #2B**).

Supplemental proposal 1 Objectives:

Supplemental Objective 1: To determine the pharmacokinetics of suramin delivery when administered via intramuscular injection in mice for the treatment of skeletal muscle injuries.

Supplemental Objective 2: To evaluate the beneficial effect of decorin, another antifibrosis agent, on muscle regeneration and healing and its mechanism of action.

Supplemental proposal 2 Objectives:

Supplemental Objective 1: To evaluate whether decorin's beneficial effect on muscle healing is mediated through its influence on muscle inflammation. **Hypothesis:** Decorin improves muscle healing by influencing muscle inflammation.

Supplemental Objective 2: To investigate whether decorin's beneficial effect on muscle healing is mediated through MSTN or FLST, two important regulators of muscle growth. **Hypothesis:** Decorin promotes muscle healing by down-regulating MSTN or up-regulating FLST.

Supplemental Objective 3: To evaluate whether angiotensin II receptor blockade after injury represents a potential non-invasive approach to improve muscle regeneration and repair after military-related muscle injury
Hypothesis: Angiotensin II receptor blockade will improve muscle healing after injury.

Relevance: These studies should further our understanding of the muscle healing process, facilitate the identification of new techniques to promote efficient muscle healing, and contribute to the development of innovative therapies for various types of muscle injuries and diseases, such as muscular dystrophies.

Project #2

Background: Muscle injuries are very common musculoskeletal problems in traumatology that arise frequently in military training and combat. Injured skeletal muscle undergoes a natural process of regeneration; however, fibrosis—the formation of fibrous scar tissue—hinders this process and precludes the complete recovery of

muscle function. Prevention of fibrosis could improve injured skeletal muscle healing; however, it often is not possible to treat muscle injuries before fibrosis occurs.

Objective/Hypothesis: We hypothesize that matrix metalloproteinase type 1 (MMP1), a collagen-digesting enzyme, can improve the microenvironment for muscle regeneration and accelerate healing by digesting existing scar tissue within recovering muscle. Further we hypothesize that MMP-2 can enhance muscle cells migration and regeneration, reorganize muscle structure and accelerate muscle healing after injury.

Specific Aims/Study Design: First, we will culture muscle-derived fibroblasts and myogenic cells in vitro in the presence of MMP1 and assess its effects on fibroblast proliferation and collagen deposition (**Technical Objective #1A**) and myogenic cell migration and differentiation (**Technical Objective #1B**). We then will inject MMP1 directly into normal (noninjured) skeletal muscle to determine the safest and most effective dose of MMP1 in vivo (**Technical Objective #2A**). Next, we will evaluate the ability of MMP1 to digest fibrous scar tissue present within injured skeletal muscle and, by so doing, to enhance the regeneration and functional recovery of injured skeletal muscle (**Technical Objective #2B**). Finally, we will attempt to extend the effective half-life of MMP1 in fibrous scar tissue by genetically engineering myoblasts and muscle-derived stem cells (MDSCs) to express MMP1 (**Technical Objective #3**).
Supplemental Proposal 1 Objectives:
We will evaluate the effects of MMP-2 on myogenic cell migration and differentiation *in vitro* (**Technical Objective #1**). We then will use MMP-2 enhance muscle cells migration and regeneration to improve the functional recovery of injured skeletal muscle *in vivo* (**Technical Objective #2**). Further, we will attempt to assess the relationship between MMP-1 and MMP-2 during muscle healing (**Technical Objective #3**).

Relevance: The study results generated by the proposed project should shed further light on the effects of scar tissue on muscle healing after injury and could facilitate the development of methods by which to eliminate scar tissue and enhance the regeneration of muscle damaged by military- or sports-related injuries or diseases.

Project #3
Our preliminary studies point to a close developmental relationship between vascular endothelial cells and myogenic cells in the adult human muscle. We have, indeed, characterized two novel populations of muscle-derived, non-satellite cells that exhibit dramatic myogenic potential in culture and *in vivo:* genuine vascular endothelial cells and cells co-expressing markers of both myogenic and endothelial cell lineages, which we have named myo-endothelial progenitors. Both cell populations can be purified by flow cytometry as endothelial (CD56- CD 34+ CD144+) and myo-endothelial (CD56+ CD34+ CD144+), and cultured for several weeks without losing their myogenic potential. In our preliminary experiments, the myogenic potential in vivo of myo-endothelial progenitors is dramatically higher than that of endothelial cells, which are themselves much more efficient than regular myogenic cells. We propose experiments to further assess the respective myogenic potentials of human muscle-derived endothelial and myo-endothelial cells, whch will be compared qualitatively and quantitatively following injection into the injured SCID mouse muscle. Besides myogenesis, the development of other cell types, notably endothelial cells and pericytes, upon intramuscular injection will be examined. We hypothesize that upon myo-endothelial cell transplantation, high rates of donor cell survival and/or proliferation will promote skeletal muscle regeneration by generating more donor cells that can subsequently participate in the regeneration process. We propose a set of experiments to determine the rate at which these different populations of human muscle-derived cells survive and proliferate at different time points after injection, and to evaluate if such differences might affect the regeneration capacity of the cells after transplantation into the skeletal muscle. We will also perform experiments to investigate if these various human muscle-derived cell populations implanted in mice *via* intramuscular injection undergo self-renewal. We will also use a previously described protocol to determine if the donor-derived muscle precursor cells isolated from the skeletal muscle of primary recipient mice can be re-transplanted into the skeletal muscle of secondary recipients and improve skeletal muscle regeneration. To this aim, we will retrovirally modify human-derived

myogenic, endothelial and myo-endothelial cells to express GFP. This will enable us to rapidly count the GFP-expressing cells isolated from primary recipients and sort them with high purity for re-transplantation into secondary recipients. Finally, we will also check if cell fusion between donor cells or between donor and host cells plays a significant role in muscle regeneration by these different subsets of human cells.

Supplemental Proposal 1 Objectives:

1- Characterize the potential of pericytes to give rise to a full array of differentiated cells following transplantation into the injured skeletal muscle. We will determine, in a xenochimeric model, whether pericytes can, besides myofibers, regenerate other cell compartments that are fundamental to wound healing and tissue regeneration: blood vessels, connective tissues, nerves.
2- Determine the potential of pericytes to give rise to more primitive stem cells (auto-renewal), and/or to committed muscle progenitor cells (satellite cells) on transplantation into the injured skeletal muscle. Long-term and iterative transplantation experiments will be performed in order to understand whether pericytes can not only regenerate skeletal muscle, but do it permanently. This is a key point to be taken in consideration in the perspective of transplanting these cells into patients.
3- Master the long-term culture of pericytes while maintaining their developmental potential intact. The biology of pericytes *in vitro* will be scrutinized in order to validate culture protocols allowing to obtain large lots of transplantable cells.

Project #4

Background: As progress toward understanding the basic biology of stem cells continues to grow, it is of vital importance that researchers maintain a focus on therapeutic applications of this technology by investigating preclinical models. Members of our laboratory have identified a mouse muscle-derived stem cell (MDSC) population that exhibits a highly enhanced ability to regenerate skeletal muscle in a muscular dystrophy model. Transplantation of this cell population results in significantly more efficient regeneration of skeletal muscle fibers and a significantly larger area of regeneration than does myoblast transplantation, a therapy that has already been tested in human clinical trials in both the United States and Canada. The isolation and transplantation of the human equivalent of these mouse MDSCs likely would improve the outcome of cell therapy for muscular disease and injury, including injuries frequently sustained by military personnel.

Objective/Hypothesis: The objectives of this project are 1) to identify the human stem cell populations that promote the most efficient skeletal muscle regeneration in a preclinical mouse model of muscle regeneration and Duchenne muscular dystrophy (DMD) and 2) to identify the optimal conditions under which to expand cell populations and obtain therapeutically relevant doses.

Study Design: We will screen human muscle-derived cells for a molecular and behavioral profile that correlates with efficient skeletal muscle regeneration in a preclinical model of DMD (Technical Objective #1). We also will identify the cell culturing conditions that best facilitate expansion of the potent populations (to therapeutically useful quantities) while maintaining the cells' phenotype and regeneration efficiency (Technical Objective #2).

Supplemental Proposal 1 Objectives: While ongoing projects are focused on characterizing how to identify and maintain the stem cell phenotype of human MDSC, we are also initiating studies to identify optimal conditions for the cryostorage of these cells. We will build on the Live cell imaging (LCI) technology as a tool identify the optimal conditions for the processing of the cells as a biologic product for therapeutic applications. LCI provides live viewing of the behavior of a stem cell population in culture and allows us to obtain numerous real-time measurements at the single cell level. The result is a detailed behavioral phenotype of the stem cell population which can be linked to in vivo outcome measures. We will use this tool to follow the changes of cell phenotype in response to temperature changes and fluctuations.

Relevance: This project represents a critical step in cell therapy: Screening potential cell candidates and studying expansion kinetics and limits to generate stem cells for use in cell and gene therapy. This project is

unique in that it takes the next step in moving muscle stem cells toward clinical application by investigating how to develop a safe and standardized approach by which to expand stem cell populations. The stem cell screening and expansion techniques that we plan to develop will facilitate the use of cell and gene therapy for myriad musculoskeletal injuries and diseases.

Project # 5

Congenital muscular dystrophy (CMD) is a group of severe forms of muscular dystrophy leading to early death in human patients. The majority of cases are caused by genetic mutations in the major laminin that contains the α2 chain (formerly named merosin) in the muscle basement membrane. The early morbidity/fatality and the lack of effective treatment require urgent search for novel therapeutics. Previously, we utilized mini-agrin, which has been proven to have a therapeutic effect in transgenic MCMD mice, to treat MCMD mice by AAV vector. Our preliminary studies showed that over-expression of mini-agrin protein by AAV vector greatly improved general health and muscle morphology in MCMD mice. However, the treated disease mice still developed gradual paralysis and displayed shorter life span than wild type mice. To further improve the current gene therapy paradigm, with the advanced AAV technology and muscle biology knowledge, we will vigorously test our hypothesis: whether muscle pathogenesis can be improved by inhibition apoptosis or promoting muscle growth. The specific aims are the following:

Aim1: To investigate whether muscle pathogenesis can be improved by delivery of BCL2, an anti-apoptotic gene, by AAV vector in MCMD mice. Mice that lack laminin α2 show severe muscle loss, poor regeneration, and a greatly shortened lifespan. A role for apoptosis in pathology of laminin α2-deficiency has been suggested by histological and in vitro studies, as well as transgenic studies. In this study, we will explore the potential therapeutic effect by delivering AAV-BCL2 vector into MCMD mice.

Aim2: To examine whether therapeutic effect can be obtained by delivery of insulin like growth factor 1 (IGF-1) gene, which can promote muscle growth, by AAV vector in MCMD mice. The MCMD mice show muscle atrophy and enhanced fibrosis as seen in human patients. Genes that promote muscle growth and inhibit fibrosis is theoretically beneficial for congenital muscular dystrophic muscle. Myostatin blockade, one of the strategies to promote muscle growth, has been shown to have a severe side effect of increasing postnatal lethality in MCMD transgenic studies. The reason for the side effect is due to significantly less brown and white fat in the absence of myostatin. In our preliminary studies, we observed that myostatin blockade significantly increased muscle weight, as well as decreased fat tissue in normal mice. However, over-expression of IGF-1 in normal mice only increased muscle weight without loosing fat. Considering less fat will result a severe side effect, we will deliver IGF1 gene to MCMD mice by AAV vector to study whether a therapeutic effect can be achieved in this proposal.

Upon completion, this project will establish complementary therapeutic strategies to combat the severe congenital muscular dystrophy in animal model, setting the base for the development of a clinically efficacious gene therapy strategy.

Project # 6

Background: Elegant studies show that certain cytokines trigger the activation of nuclear factor kappaB (NF-kB) mediated by phosphorylation and degradation of the NF-kB inhibitory protein, IkappaBalpha. Downstream effects of pathological NF-kB activation in skeletal muscle include the inhibition of new muscle formation and the degeneration of existing muscle. *In vitro* studies support the potential that the IkappaBalpha superrepressor (IKBSR), an IkappaBalpha genetically engineered to prevent its phosphorylation, can prevent the activation of NF-kB in skeletal muscle and could ameliorate or prevent muscle wasting. Our preliminary studies demonstrate the novel determination of inhibition of activation of NF-kB by cFLIP.

Objective/Hypothesis: The proposal will explore the mechanism of muscle wasting in an *in vitro* model and develop novel gene transfer vehicles testing the hypothesis that gene transfer strategies can promote muscle regeneration toward a goal of improving muscle bulk and strength in the setting of injuries or diseases that cause muscle atrophy.

Specific Aims:

Aim 1: To characterize an *in vitro* model of cancer-induced muscle wasting in primary muscle cells and in stable muscle cell lines expressing IKBSR or cFLIP.
Aim 2: To clone, rescue, and purify AAV serotype 1 vectors carrying IKBSR or cFLIP and characterize expression and function *in vitro* in anticipation of future use in an *in vivo* model of muscle wasting.

Study Design: We will characterize an *in vitro* model of muscle wasting studying NF-kB activation, the ubiquitin-proteasome system and caspase activation. We will test whether genetic modifications to muscle cells will confer a benefit by blocking pathways of muscle degeneration. We will develop gene transfer vectors designed to promote muscle regeneration.

Supplemental Proposal 1 Objectives:
Supplemental Objective 1: To test gene transfer approaches in an *in vivo* model of cachexia
A. Test AAV carrying IKBSR for treatment of cancer cachexia
B. Test AAV carrying cFLIP for treatment of cancer cachexia
Supplemental Objective 2: To test peptide transduction approaches of inhibiting NF-κB activation as treatment for muscle cachexia
A. Test peptide transduction in an *in vitro* assay of muscle cachexia
B. Test peptide transduction in an *in vivo* assay of muscle cachexia

Relevance: The ability to promote muscle regeneration in the setting of focal or generalized muscle loss could confer significant clinical benefit in the setting of focal neuropathic or other processes that cause muscle atrophy or chronic illnesses that cause cachexia.

Vector Core:
Background:
The newly established molecular biology laboratory (MBL) will be a Vector Core for the project "Molecular Therapy of Muscle Repair". The goal of this core is to construct vectors such as adeno-associated-, adenoviral- and retoviral vectors for this project. Besides the tissue specific promoter and optimal serotype of vectors in the project #5 and project #6, we will develop a newly modified AAV vector, called the Tet/on and Tet/off AAV vector. This new vector is very useful to ameliorate the acute inflammatory during the early time of muscle injuries. The gene in Tet/on vector will be induced to express the therapeutic gene in the presence of doxycycline, an antibiotic. To avoid the toxicity caused by gene over expression, the vector will be turned off at the time when the muscle is healed.
Objectives:
Objective 1: To continue the production of AAV vectors for the following genes
A: Decorin for project #1
C: Mini-dystrophin gene
D: MPRO (myostatin propeptide) for project # 5
Objective 2: To construct the following genes into AAV vector
A: MMP1 for project #2
B: IKBSR for project #6
C: cFLIP for project #6
Objective 3: To design the new AAV vector containing muscle tissue specific promoter.
Objective 4: To develop the adenoviral and retroviral vectors.

BioReactor Core
The Bioreactor Core serves as a resource for project investigators in need of dynamic cell population analyses. This core uses the Automated Cell CytoWorks™ (ACCW) robotic system to characterize and compare various populations of adult-derived stem cells. The system, in theory, can give the investigator a read out of 20 to 30 different parameters in the form of a "Phenoprint©". We have also planned to set up internet technology to share the information generated in the core facility and thus facilitate further study and development of adult-derived stem cell–based therapies by members of the broader scientific community.

Project # 1
Progress Report
The use of suramin to improve muscle healing after military-related muscle injuries

(Johnny Huard)

Introduction

Muscle injuries, especially pulls and strains, are among the most common and most frequently disabling injuries sustained by athletes and soldiers. Although injured muscles heal naturally, the regeneration is very slow and often yields incomplete functional recovery. In injured muscle, regeneration begins shortly after injury, but the healing process is rather inefficient and is hindered by fibrosis—that is, scar tissue formation. More importantly, the scar tissue that often replaces damaged myofibers may contribute to the tendency of strains to recur. We have observed that TGF− β1 plays a central role in skeletal muscle fibrosis and, more importantly, that the use of antifibrosis agents that inactivate this molecule, such as suramin (a Food and Drug Association [FDA]-approved drug that prevents fibrosis due to skin disorders), can reduce muscle fibrosis and consequently improve muscle healing, resulting in nearly complete recovery after laceration or strain injuries.

Body

Original Proposal

Muscle injuries are very common musculoskeletal problems, and the experiments described in this application are highly relevant to currently unmet medical needs. Although we do not yet understand all the events required for efficient muscle healing, enough information exists for us to vigorously test the proposed research aims. Our extensive preliminary results, presented below, strongly suggest that researchers working to develop biological approaches to improve muscle healing after injury should focus on the elimination of fibrosis within injured muscle tissue. We have observed that TGF-β1 plays a central role in skeletal muscle fibrosis; the use of antifibrosis agents that inactivate this molecule can reduce muscle fibrosis and consequently improve muscle healing after injury. Although the effect of TGF-β1 can be blocked in various ways—including treatment with a neutralizing TGF-β1 antibody, the antisense and ribozyme to TGF- 1, decorin, IFN- and - , or relaxin—the ability of suramin to neutralize the fibrotic effect of TGF-β1 and enhance muscle regeneration makes this molecule particularly well-suited for use in applications to improve muscle healing after injury. In addition, the fact that suramin is already approved by the FDA for use in humans makes this molecule an ideal candidate for use in clinical applications to improve muscle healing in soldiers. Using an animal model of muscle contusion, a common military injury, we will determine the appropriate dose and time of administration of suramin (**Technical Objective 1**). Better characterization of the mechanisms by which suramin enhances muscle regeneration is extremely important because an improved understanding of these mechanisms may reveal novel ways to promote muscle growth and regeneration (**Technical Objective 2A**). We also will investigate suramin's interaction with myostatin and follistatin, 2 important regulators of muscle growth (**Technical Objective 2B**). In summary, the studies proposed in this application will enable us to determine whether suramin, a drug already approved by the FDA, can improve muscle healing by preventing scar tissue formation after a common military injury (muscle contusion); we also will examine the mechanism(s) by which suramin promotes muscle regeneration. The results of these studies could aid in the development of innovative therapies to promote healing in muscles damaged by different types of injury or disease (e.g., muscular dystrophies), therapeutic approaches that could easily be translated from animal experiments to human clinical applications.

The overall goals of the proposed project are as follows: (i) demonstrate that suramin can improve muscle healing in a dose- and time-dependent manner by preventing scar tissue formation after muscle contusion (a common military injury); (ii) determine the mechanisms by which suramin

enhances muscle healing after injury. We will investigate whether the enhanced muscle healing observed after suramin treatment is due solely to its inhibition of TGF-β1 activity (**Technical Objective 1)** or to suramin's promotion of muscle regeneration through potential interactions with muscle growth regulators, particularly its possible down-regulation of myostatin or up-regulation of follistatin (**Technical Objectives 2A and 2B**).

Supplemental Proposal 1

New Technical Objective 1*:* To determine the pharmacokinetics of suramin delivery when administered via intramuscular injection in mice for the treatment of skeletal muscle injuries.

Our laboratory has shown that intramuscular (IM) injection of suramin after laceration and strain injuries decreases skeletal muscle fibrosis in mice and improves muscle healing, resulting in a near complete recovery of muscle function (Chan et al, 2002; Chan et al, 2003). The clinical availability of suramin makes this agent particularly appealing for its use in the treatment of muscle injuries in humans. However, to date, clinical investigations of the use of suramin have been primarily limited to intravenous administrations, which is complicated by a narrow therapeutic index, and dose-limiting toxicities. The dose-limiting toxicities are related to suramin plasma concentrations, of which acceptable levels and durations for humans have been previously reported (Eisenberger et al, 2005). With this in mind, we will perform pre-clinical pharmacokinetic studies in mice to evaluate the concentration/ time data of suramin when administered intramuscularly. Specifically, we will perform measures of therapeutic dosing, half-life and clearance calculations, which will then be used as a guide for the design of phase 1 clinical trials.

New technical objective 2*:* To evaluate the beneficial effect of decorin, another antifibrosis agent, on muscle regeneration and healing and its mechanism of action.

As mentioned above, various reports indicate that the overproduction of TGF-β1 in response to injury and disease is a major cause of tissue fibrosis in animals and humans. We have observed that TGF-β1 plays a central role in skeletal muscle fibrosis and that the use of antifibrosis agents to inactivate this molecule can reduce muscle fibrosis and consequently significantly improve muscle healing after injury. Although the effect of TGF-β1 can be blocked by various approaches—including treatment with a neutralizing TGF-β1 antibody, the antisense and ribozyme to TGF-β1, suramin [The focus of the last year research proposal], relaxin, and IFN-γ —the unique ability of decorin to neutralize the fibrotic effect of TGF-β1 and independently enhance muscle regeneration makes this molecule particularly well-suited for use in applications to improve muscle healing after injury. Our preliminary data demonstrate that the stimulation of myogenic cells (C2C12 cells) with decorin enhances myogenic differentiation in vitro. Similarly, C2C12 cells genetically engineered to express decorin display an enhanced ability to undergo myogenic differentiation in vitro and in vivo. We also have observed that direct gene transfer of decorin by injection into uninjured skeletal muscle can decrease the fibrosis and enhance the muscle regeneration observed after subsequent injury of that muscle. These results suggest that decorin may independently enhance muscle regeneration and impede fibrosis. On the basis of our prior and preliminary findings, we propose to validate the beneficial effect of decorin on muscle regeneration by conducting gain- and loss-of-function experiments.

Supplemental Proposal 2

Additional Technical Objective #1: To evaluate whether decorin's beneficial effect on muscle healing is mediated through its influence on muscle inflammation.

Hypothesis: Decorin improves muscle healing by influencing muscle inflammation.

It has been observed that decorin influences the differentiation, survival, and infiltration of macrophages. We, therefore, propose experiments designed to reveal the role of decorin on the inflammatory phase of muscle healing. To determine the influence of decorin on the infiltration of inflammatory cells (macrophages and neutrophils) within the injured skeletal muscle, we will conduct gain- and loss-of-function experiments involving

the injured skeletal muscles of decorin-/- mice, decorin-over-expressing mice (after AAV gene transfer of decorin), and normal WT mice as described above. To conduct the gain-of-function experiments, we will use gene therapy (adeno-associated viral [AAV] gene transfer in collaboration with Dr. Bing Wang; Vector Core) to deliver decorin into lacerated skeletal muscle (decorin-expressing mice). We will use decorin-/- knockout mice (provided by Dr. Renato V. Iozzo) to perform the loss-of-function experiments. If decorin is observed to have an effect on inflammatory cells, we will subsequently determine whether the COX-2-dependent prostaglandins (PGs) are also modulated by decorin since it has been shown by our laboratory as well as others that the COX-2 pathway is essential during the early stage of skeletal muscle regeneration. If the COX-2 pathway is influenced, at least in part, by decorin, we will use COX-2 deficient mice. as well as clodronate liposomes to deplete macrophages and determine whether the beneficial effect of decorin on muscle healing is related to decorin's effect on muscle inflammation.

Additional Technical Objective #2: To investigate whether decorin's beneficial effect on muscle healing is mediated through MSTN or FLST, two important regulators of muscle growth.

Hypothesis: Decorin promotes muscle healing by down-regulating MSTN or up-regulating FLST.

The first set of experiments will enable us to determine whether differences exist among the levels of MSTN and FLST expression in the injured skeletal muscles of decorin-/- mice, decorin-overexpressing mice (mice with their skeletal muscle transduced with AAV-decorin), and normal wild-type (WT) mice. We will measure the levels of MSTN and FLST expression in the injured skeletal muscle at different times after injury. We will compare the results in the lacerated muscle of normal WT mice with those obtained from the lacerated muscle of decorin-/- mice and mice whose muscles overexpress decorin. The observation that the level of MSTN and FLST expression in injured skeletal muscle differs between these different mice (decorin-/-, decorin++, and normal mice) will support the hypothesis that decorin interacts with MSTN or FLST.

The second set of experiments will evaluate the effect of decorin on the healing of lacerated muscles in myostatin-overexpressing mice, which typically display lower muscle mass and reduced muscle size relative to normal WT mice [66]. We will compare the results with those observed in the lacerated muscles of MSTN-/- mice and normal mice treated with decorin. We then will measure the level of muscle regeneration and healing at different times after injection. A reduction in the beneficial effect of decorin on muscle healing in the MSTN++ mice, when compared to the injection of decorin in normal and MSTN-/- mice, will support our preliminary findings that MSTN interferes with the beneficial effect of decorin in muscle regeneration.

In a third set of experiments, we are proposing to investigate whether the detrimental effect of MSTN observed in normal mice is influenced by decorin expression. We will evaluate the effect of MSTN on the healing of lacerated muscles in decorin-overexpressing mice (after AAV gene transfer of decorin). We will compare the results with those observed in the MSTN-treated lacerated muscles of decorin-/- mice and normal WT mice. We then will measure the level of muscle regeneration and healing at different times after injection. We will compare the results with the healing observed in the lacerated muscle of decorin-/- mice and normal muscle treated with the same dose of MSTN. A reduction on the detrimental effect of MSTN on muscle healing in the decorin++ mice, when compared to the decorin-/- and normal muscle, will support our preliminary findings that decorin interferes with the deleterious effect of MSTN in skeletal muscle.

We anticipate that the beneficial effect of decorin on muscle healing will be influenced by the level of MSTN expression and will be significantly reduced in the MSTN++ mice. Similarly, the detrimental effect of MSTN on muscle healing will also be influenced by the level of decorin expression and will be significantly reduced in the decorin over-expressing mice. We alternatively propose a ***final set of experiments*** to determine whether decorin interacts either directly with MSTN or potentially through an intermediate molecule such as FLST, another muscle regulator. In fact, research has shown that mice overexpressing FLST display a dramatic increase in muscle mass, and that FLST antagonizes MSTN by a direct protein interaction. Indeed, our preliminary results show that FLST-treated muscle cells differentiate into myotubes that are significantly larger than those formed by nontreated cells, and decorin up-regulates the expression of FLST in muscle cells *in vitro*. These results support the hypothesis that

decorin's beneficial effect on muscle healing is related to the up-regulation of FLST. Therefore, we will examine the effect of FLST on muscle regeneration and healing after laceration injury in decorin++ mice (after AAV decorin gene transfer) and compare the results with those observed in normal WT mice and decorin-/- mice. We then will measure the level of muscle regeneration and healing at different times after injection, and compare the results among the different groups at different times after injury. A reduction in the beneficial effect of FLST on muscle healing in the decorin-/- mice, when compared to the WT and decorin++ mice, will support our preliminary findings that decorin also interacts with FLST. These results, in combination with the fact that decorin up-regulates the expression of FLST in muscle cells *in vitro*, will support the contention that decorin improves muscle healing through a potential up-regulation of FLST which will consequently antagonize the effect of myostatin.

Additional Technical Objective #3: To evaluate whether angiotensin II receptor blockade after injury represents a potential non-invasive approach to improve muscle regeneration and repair after military-related muscle injury

Hypothesis: Angiotensin II receptor blockade will improve muscle healing after injury.

In our laboratory, we have investigated several biological agents, which have proven to be of some benefit in altering the natural course of muscle injury. Specifically, we have focused our recent efforts on agents that inhibit muscle fibrosis via inhibition of TGF-β1, a key cytokine in the fibrotic signaling pathway in skeletal muscle . Using decorin, suramin, relaxin, and gamma interferon, we have demonstrated that these therapies can decrease fibrosis and increase regeneration following skeletal muscle injury. However, their use clinically is hampered by relatively severe side-effect profiles, lack of oral dosing formulations, and, in the case of decorin, lack of FDA approval for use in humans. These limitations are under investigation as part of our current line of funding from the Department of Defense (DOD).

Fibrosis is a pathological process that is not unique to the skeletal muscle system. Observations have linked pathologic fibrosis in various organ systems to the local effects of a naturally-occurring molecule, angiotensin II, an end-product of the blood pressure-regulating renin-angiotensin system. The modulation of angiotensin II with angiotensin converting enzyme inhibitors or angiotensin II receptor blockers has demonstrated decreased fibrosis and improved function in liver, kidney, and lung tissue. Injured cardiac muscle in disease entities such as congestive heart failure also demonstrates dysfunction related to fibrosis. Myocardium exposed to decreased levels of angiotensin II, either through the use of angiotensin converting enzyme inhibitors or angiotensin receptor blockers, has also demonstrated measurably improved function. Investigators have also observed a relationship between the modulation of angiotensin II and skeletal muscle. Patients treated with angiotensin-modulating medications for hypertension also displayed the unexpected side-effect of decreased rates of muscle wasting and a reduction in the relative amount of adipose tissue within their musculature. Moreover, elite athletes, particularly those in endurance sports, have inherent differences in their body's metabolism of angiotensin II as decreased exposure resulted in improved skeletal muscle function.

Recent experiments have been performed to elucidate the mechanism by which angiotensin II receptor blockade modulates TGF- β1, which has also been implicated in the prevention of muscle regeneration in murine models of chronic myopathic disease. We then hypothesized that systemic delivery of an angiotensin II receptor blocker may enhance muscle regeneration and decrease fibrosis in a laceration model of skeletal muscle injury. To test this hypothesis, we performed a set of experiments where forty mice underwent bilateral partial gastrocnemius lacerations. Mice were assigned randomly to a control group (tap water), a low-dose angiotensin receptor blocker (Losartan, Merck) group (30 mg/Kg/day), or a high-dose angiotensin receptor blocker group (300 mg/Kg/day). Mice were sacrificed 3 or 5 weeks after injury, and the lacerated muscles were examined histologically for myofiber regeneration and fibrosis. *In vitro* experiments measured the production of TGF- β1 induced by angiotensin II in a cell line of fibroblasts through ELISA and western blot. A second set of experiments were performed for physiological testing to establish the peak force and torque generated by the recovering muscle in each group.

Our results indicated that angiotensin II induced the expression of TGF-β1 in fibroblasts. Compared

with control mice at 3 and 5 weeks, Losartan-treated mice (both low and high-dose groups) exhibited a significant and dose-dependent improvement in muscle regeneration and a measurable reduction in scar tissue formation within the area of injury. The treatment groups showed a trend toward functional improvement after five weeks of therapy.

These results indicate that by modulating the response to local and systemic angiotensin II, angiotensin receptor blocker therapy significantly reduced fibrosis and led to an increase in the number of regenerating myofibers in acutely injured skeletal muscle. These effects were apparent as early as 3 weeks after injury in mice treated with one-tenth of the anti-hypertensive dose of angiotensin receptor blocker used clinically. The clinical implications for this application of angiotensin receptor blockers are potentially far-reaching and include not only sports and military-related injuries, but also diseases like the muscular dystrophies. We believe that this treatment could be effective when instituting a non-invasive treatment after the injury has taken place, a more clinically relevant scenario in treating muscle injuries.

Results of Original Technical Objective:

It was unknown whether suramin could improve muscle healing after contusion injury, the most commonly encountered muscle injury. Past studies had been performed on mice that had received laceration and strain injuries It also remains unclear whether this enhanced muscle regeneration is a direct effect of suramin. We have performed studies during the first year of funding to examine whether suramin would promote differentiation of myogenic cells *in vitro* and improve injured muscle healing by enhancing regeneration and reducing fibrosis *in vivo*, by using an animal model of muscle contusion.

Muscle-derived stem cell differentiation assay: Muscle-derived stem cells (MDSCs) were isolated from wild type mice (C57BL/6J) via the modified preplate technique. MDSCs (10^4 cells/well) were seeded into 12-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 10% horse serum, 0.5% chicken embryo extract, and 1% penicillin/streptomycin. After 24 hours, the medium was replaced with differentiation medium (DMEM containing 2% horse serum and 1% penicillin/streptomycin) containing different concentrations of suramin (0, 1, 10, and 100 μL/mL). After another 24 hours, the medium was replaced with differentiation medium. All cells were grown at 37^{o}C in 5% CO_2. Three days after incubation, the fusion index was assessed by counting the number of nuclei in differentiated myotubes as a percentage of the total number of nuclei.

Immunocytochemistry in vitro: Immunocytochemistry was performed on the cells *in vitro* to examine their expression of fast myosin heavy chain (MyHC

Animal model: The muscle contusion model was developed in normal wild-type mice (7 to10 weeks of age, with an average weight of 24.0g). A 17g stainless steel ball was dropped through an impactor from a height of 100 cm onto the animal's tibialis anterior (TA) muscle. Mice were divided into 4 groups (5 mice/group). Different concentrations of suramin (0, 2.5, 5, and 10 mg in 20 μL of phosphate-buffered saline [PBS]) were injected intramuscularly two weeks after injury. Statistical analysis was performed with ANOVA.

Suramin stimulates MDSC differentiation: Suramin treatment promoted the myogenic differentiation of MDSCs *in vitro* in a dose-dependent manner. We observed a significantly higher fusion index in each of the two suramin treatment groups (10 and 100 μg/mL**)** than in the control group (0 μg/mL). Furthermore, 100 μg/mL of suramin treatment enhanced the differentiation significantly more than the other suramin treatments (1 and 10 μg/mL).

Suramin enhances muscle regeneration and decreases fibrosis after contusion injury: We observed a significant increase in the number of regenerating myofibers in all of suramin treated groups (2.5, 5, and 10 mg/20 μL PBS) when compared with the control group (0mg/20μL of PBS).

Moreover, Masson's trichrome staining showed significantly less fibrotic area in all of suramin treated groups than in the control group. Although all three suramin treated groups showed significant improvement in healing by way of muscle regeneration and fibrosis inhibition, there was no significant difference between the three suramin treatment groups.

This data indicate that the suramin-treated MDSC groups have higher fusion indices than the control group *in vitro*This suggests that suramin can enhance the myogenic differentiation of MDSCs, and reveals the potential mechanism by which suramin enhances muscle regeneration following injury. This is the first study to show that suramin not only has antiproliferative effects on fibroblasts, it affects the myogenic differentiation of MDSCs directly. Our results clearly indicate that suramin can enhance muscle regeneration and prevent fibrosis after a contusion injury, the most common muscle injury **(Refer to Appendices 1, 7 and 8)**. Our future study will investigate the mechanism(s) by which suramin enhances the myogenic differentiation of myogenic cells.

We have recently furthered these findings by performing a series of experiments to determine the mechanism (s) behind the beneficial effect of suramin on muscle healing after injury. Our hypothesis was that suramin enhances muscle healing by both stimulating muscle regeneration and preventing fibrosis in contused skeletal muscle. *In vitro*: Myoblasts (C2C12 cells) and muscle-derived stem cells (MDSCs) were cultured with suramin and the potential of suramin to induce their differentiation was evaluated. Furthermore, MDSCs were co-cultured with suramin and myostatin (MSTN) to monitor the ability of suramin to neutralize the effect of MSTN. *In vivo*: Varying concentrations of suramin were injected in the tibialis anterior muscle of mice two weeks after muscle contusion injury. Muscle regeneration and scar tissue formation were evaluated by histological analysis and functional recovery was measured by physiological testing Our results demonstrated that suramin stimulated the differentiation of myoblasts and MDSCs in a dose-dependent manner. Moreover, suramin neutralized the inhibitory effect of MSTN on MDSC myogenic differentiation. *In vivo*, suramin treatment significantly promoted muscle regeneration, decreased fibrosis formation, reduced myostatin expression in injured muscle, and increased muscle strength after contusion injury.
In conclusion our results indicate that intramuscular injection of suramin following a contusion injury improved overall skeletal muscle healing. Suramin enhanced myoblast and MDSC differentiation and neutralized MSTN's negative effect on myogenic differentiation *in vitro*, which suggests a possible mechanism for the beneficial effects that this pharmacological agent exhibits *in vivo*. We believe that these findings could contribute to the development of biological treatments to aid in muscle healing after experiencing a muscle injury. These results were recently published in the American Journal of Sports Medicine (**refer to Appendix 8**).

In a final set of experiments, we have examined whether suramin treatment enhances muscle regeneration and reduce fibrosis by down-regulating myostatin expression *in vivo*. The muscle contusion was made on the tibialis anterior (TA) muscle of each mouse. Two weeks after injury, different concentrations of suramin (0 and 2.5 mg) were injected intramuscularly (n=20 mice/ group). At different time points (0.5, 1, 2, 10, and 14 days after injection), mice were sacrificed and cryosections of TA muscle were analyzed histologically. Suramin (2.5 mg) injection demonstrated a significant increase in the number of regenerating myofibers and reduction of fibrotic area when compared with the control group (0 mg). Furthermore, suramin injection effectively inhibited the expression of myostatin in the injured muscle. Our results suggest that suramin improves skeletal muscle healing by enhancing regeneration and reducing fibrosis after contusion injury through a potential decrease in myostatin expression in the injured skeletal muscle. Our findings may contribute to the development of progressive therapies for muscle injury. (**Refer to Appendices 1, 7 and 8)**

Results of Supplemental Proposals 1 and 2:

We have shown that decorin, a small leucine-rich proteoglycan, can inhibit TGF-β1 to prevent fibrous scar formation and improve muscle healing after injury. In the decorin-treated muscle, an enhancement of muscle regeneration is observed through histological examination. We have recently determined whether decorin has a direct effect on myogenic cells' differentiation. Our results indicate that myoblasts genetically engineered to express decorin (CD cells) differentiated into myotubes at a significantly higher rate than did control myoblasts (C2C12). This enhanced differentiation led to the up-regulation of myogenic genes (*Myf5*, *Myf6*, *MyoD*, and *myogenin*) in CD cells *in vitro*. We speculate that the higher rate of differentiation exhibited by the CD cells is due to the up-regulation of follistatin, PGC-1α, p21, and the myogenic genes, and the down-regulation of TGF-β1 and myostatin. Decorin gene transfer *in vivo* promoted skeletal muscle regeneration and accelerated muscle healing after injury. These results suggest that decorin not only prevents fibrosis, but also improves muscle regeneration and repair. (**Refer to Appendix 2**)

Recent studies have shown that myostatin (MSTN), first identified as a negative regulator of skeletal muscle growth, may also be involved in the formation of fibrosis within skeletal muscle. In a recent study, we further explored the potential fibrotic role of MSTN, as well as its interactions with both transforming growth factor-beta1 (TGF-β1) and decorin. We discovered that MSTN stimulated fibroblast proliferation in vitro, and induced its differentiation into myofibroblasts. We further found that, while TGF-β1 stimulated MSTN expression, MSTN stimulated TGF-β1 secretion in C2C12 myoblasts. Decorin, a small leucine-rich proteoglycan, was found to neutralize the effects of MSTN in both fibroblasts and myoblasts, and up-regulate follistatin (FSTN), an antagonist of MSTN. Moreover, FSTN, an antagonist of MSTN, was up-regulated by decorin. The results of in vivo experiments showed that MSTN-knockout mice developed significantly less fibrosis and displayed better skeletal muscle regeneration when compared to wild-type mice at 2 and 4 weeks following laceration injury. In wild-type mice, we found that MSTN stimulated myofibers to express TGF-β1 in skeletal muscles at early time points following injection. Both TGF-β1 and MSTN were additionally seen to co-localize in myofibers in the early stages of injury. In summary, these findings define a fibrogenic property of MSTN, and indicate a coregulatory relationship between TGF-β1, MSTN, and decorin. Please refer to the references listed below in the Reportable Outcomes Section to access these results. The complete recovery of injured skeletal muscle has posed a constant challenge for orthopaedic physician. Once injured, skeletal muscle is able to undergo regeneration from satellite cells; nevertheless, in the serious injured muscle, the formation of fibrosis often impedes effective muscle regeneration and resulted in an incomplete muscle healing. Therefore, to develop biological approaches to improve muscle healing, it is crucial to better understand the mechanisms of the skeletal muscle fibrosis. In the current studies, we found that myostatin (MSTN), a member of TGF-β family, plays a role in the formation of skeletal muscle fibrosis, besides the other putative fibrosis stimulator, TGF-β1. *In vitro*, MSTN directly stimulated the proliferation of fibroblasts and their productions of fibrotic proteins. *In vivo*, after laceration injury, gastrocnemius muscles of MSTN-/- mice showed less fibrosis and better muscle regeneration than wide-type (WT) counterparts. Considering MSTN as a therapeutic target of skeletal muscle healing, we found that inhibitors of MSTN, MSTN propeptide (MPRO) and follistatin, effectively blocked MSTN signaling and improved skeletal muscle healing after injured. We used adeno-associated virus (AAV)-mediated MPRO cDNA to successfully deliver MPRO *in vivo* and improve skeletal muscle healing of normal mice after laceration, and ameliorate dystrophic pathology of *mdx*/SCID mice. Furthermore, our results demonstrated FLST overexpression (FLST/OE) mice exhibited decreased fibrosis and increased muscle regeneration in injured skeletal muscle as compared to wild-type (WT) mice. Moreover, muscle progenitor cells (MPCs) isolated from MSTN-/- and FLST/OE mice significantly regenerated more myofibers than MPCs obtained from WT mice, when transplanted into dystrophic muscles. Collectively, our results suggested that MSTN directly stimulated fibrosis in the injured skeletal muscle; blocking MSTN signaling with MPRO or FLST improved skeletal muscle healing after laceration injury; blocking MSTN signaling in donor MPCs significantly enhanced the success of cell transplantation into dystrophic muscles. Our

studies not only uncover some of the mechanisms implicated in skeletal muscle fibrosis and regeneration, and help the development of new therapeutic approach for promoting the healing of injured or diseased skeletal muscle, but also render a new sight of how to obtain robust genetically modified cell populations for cell therapy. (**Refer to Appendices 5, 11, 13**)

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although these injuries are capable of healing, complete functional recovery is hindered by the formation of dense scar tissue triggered by TGF-β1. We have previously reported that several agents such as decorin and suramin can inhibit fibrosis and improve regeneration in injured skeletal muscle. The safety of these agents, however, remains unknowns to use those agents for treating muscle injury. By contrast, Losartan (LOS), one of the Angiotensin II Receptor Blockers (ARBs)—is a FDA approved antihypertensive medication and has been shown to also be antifibrotic in a variety of tissues, including skeletal muscle. This ARB has a well-tolerated side-effect profile, can also block TGF-β1 to attenuate the development of pathological fibrosis. In this study, we investigated optimum dose of LOS in treating injured muscle to help the translation of this research from bench to bedside.
The biphasic effect of LOS on C2C12 myoblasts *in vitro*, stimulating at low dose while decreasing at high dose, suggested that there is an optimal dose of LOS. Consequently we found that LOS improves skeletal muscle regeneration at 4 weeks after contusion injury, except that these effects were reduce/eliminated in 1000 mg/kg/day group. The best effective dose was 300 mg/kg/day. Overall, these effects of LOS were more pronounced for regeneration than for fibrosis. These *in vivo* results are consistent with our *in vitro* results that LOS was able to exert effects on C2C12 whereas fibroblasts were not affected by either ANG or LOS. Regeneration and fibrosis are two competitive processes after muscle injury; therefore, decreasing fibrosis observed in LOS-treated group is can be the result of the increase regeneration. In other words, LOS might indirectly reduced fibrosis by directly stimulating regeneration. Although there were not statistical differences in fibrosis in lower dose LOS groups (3 and 30 mg/kg/day) as compared to control, these effects of LOS on both regeneration and fibrosis showed similar dose dependent trend. However, *in vivo* results above were obtained only from single time point after injury. Since there are time lags between peaks of myofiber regeneration and fibrosis after injury, further investigations are required to examine the effect of LOS on regeneration and fibrosis at their individual peak time, which will facilitate clinical application of ARBs in improving skeletal muscle healing. (**Refer to Appendices 9, 12 and 14**).

Finally and perhaps most importantly members of our clinical team in Orthopaedic surgery recently brought the use of Losartan to clinic and successfully treated a 21 year old man who had experienced a recurrent hamstring strain. After obtaining the subject's informed consent for treatment, he was started on a 30-day treatment course of losartan at the manufacturer's recommended dose of 50 mg per day by mouth. The subject underwent a routine rehabilitation supervised by his athletic trainer along with the pharmaceutical treatment, gradually progressing to eccentric strengthening. He was followed every 7-10 days with serial measurement of hamstring flexibility and strength. The subject reported no side effects while he was taking the study medication and remained normotensive throughout.
Eleven weeks after the injury, an isokinetic test of the hamstrings was performed showing an essentially normal result. In summary, all isokinetic testing parameters were within a normal range compared to the contralateral leg and well below the usual time of typically healing of a muscle injury of this type. (**Refer to Appendix 14**)

Key Research Accomplishment

- Determined that Suramin can stimulate MDSC myogenic differentiation *in vitro.*

- Determined that Suramin can enhance skeletal muscle regeneration and reduce fibrosis following a contusion injury.

- Determined that Suramin's beneficial effects on muscle healing is related to it's ability to enhance the proliferation and myogenic differentiation of myoblasts and MDSC and it's ability to inhibit myostatin and up-regulate follistatin.

- Determined that the antifibrotic ability of decorin not only relates to its ability to inhibit TGF-β1 but also neutralize myostatin. We further showed that the up or down regulation of TGF-β1 simultaneously had the same effect on myostatin and vice versa.

- Myostatin has been shown to be a negative regulator of skeletal muscle regeneration and a positive regulator of fibrosis deposition which can be down-regulated with decorin and suramin

- Follistaitin is intricately involved with the muscle healing process and has been shown to be a positive regulator of muscle regeneration and a negative regulator of fibrosis deposition and can be up-regulated with decorin and suramin..

- Losartan has been shown to block TGF-β1 and have a benefical effect on the healing of injured skeletal muscle.

- Losartan is a safe and effective, clinically applicable angiotensin II receptor blocker and has been shown to be a potential safe and effective treatment for aiding in the healing of skeletal muscle injury.

- 1st case report of treating a recurrent hamstring injury in a 21 year old man with Losatrin is being prepared and showed a beneficial effect on muscle healing.

Reportable Outcomes

1) **Nozaki M, Li Y, Zhu J, Ambrosio F, Fu FH and Huard J.** The use of suramin to improve skeletal muscle healing after contusion injury. Presented as a poster at the Orthopaedic Research Society meeting, Feb. 2007.

2) **Li Y, Li J, Zhu J, Sun B, Branca M, Tang Y, Foster W, Xiao X, Huard J**. Decorin gene transfer promotes muscle cell differentiation and muscle regeneration. Mol Ther 2007 Sep; 15(9):1616-1622.

3) **Zhu J, Li Y, Shen W, Qiao C, Ambrosio F, Lavasani M, Nozaki M, Branca M, Huard J.** Relationships between TGF-β1, myostatin, and decorin: Implications for skeletal muscle fibrosis. J Biol Chem 2007 Aug; 282(35):25852-63.

4) **Shen W, Huard J.** Tissue therapy: Implications of regenerative medicine for skeletal muscle. Attala A, Lanza R, Thomson J, Nerem RM, editors. Principles of Regenerative Medicine. Elsevier Academic Press, 2007; 1232--1247, chapter 72. (Invited Book Chapter)

5) **Zhu J, Li Y, Branca M, Huard J**. Follistatin improves skeletal muscle healing after injury. The Orthopaedic Research Society (ORS) 53rd Annual Meeting; Feb 12-14, 2007; San Diego, CA

6) **Matsuura T, Li Y, Giacobino JP, Fu F, Huard J.** Skeletal muscle fiber type conversion during the repair of mouse soleus: Potential implications for muscle healing after injury. J. Orthop Res 2007 Nov; 25(11): 1534-1540**.** Figure featured on front cover as cover art.

7) **Nozaki M, Li Y, Zhu J, Uehara K, Ambrosio F, Fu F, Huard J.** Suramin can enhance the skeletal muscle healing by blocking myostation. The Orthopaedic Research Society (ORS) 54th Annual Meeting; March 2-5, 2008; San Francisco, CA.

8) **Nozaki M, Li Y, Zhu J, Ambrosio F, Uehara K, Fu F, Huard J.** Improved muscle healing after contusion injury by the inhibitory effect of Suramin on Myostatin, a negative regulator of muscle growth. Am J Sports Med, 2008 Dec; 36(12):2354-62.

9) **Bedair H, Karthikeyan T, Quintero AJ, Li Y, Huard J.** Angiotensin II Receptor Blockade administered after injury improves muscle regeneration and decreases fibrosis in normal skeletal muscle. Am J Sports Med, 2008 Aug; (36)8: 1548-1555.

10) **Shen W, Li Y, Zhu J, Schwendener R, Huard J.** Interaction between macrophages, TGF-beta 1, and the COX-2 pathway during the inflammatory phase of skeletal healing after injury. J of Cellular Physiology 2008 Feb; 214(2): 405-412.

11) **Ambrosio, F, Li Y, Usas A, Boninger, ML, Huard J**. Muscle repair after injury and disease. Orthopaedic Biology and Medicine, Musculoskeletal Tissue Regeneration: Biological Materials and Methods. The Humana Press Inc, 2008. (Invited Review)

12) **Zhu J, Ma J, Lu A, Qiao C, Li J, Li Y, Xiao X, Huard J.** Blocking Myostatin Improves the Success of Muscle Cell Transplantation. AFIRM All Hands Meeting; January 13- 16, 2009; St. Pete, FL.

13) **Uehara K, Nozaki M, Zhu J, Quintero A, Ota S, Fu F, Huard J.** Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing in a Dose Dependent Manner. Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009.

14) **Zhu J, Ma J, Qiao C, Jianbin L, Yong L, Xiao X, Huard J**. Blocking Myostatin Improves Muscle Healing Via Enhancement of Angiogenesis. Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009.

15) **Yuri Chun, MD, James Irrgang, PhD, Tanya Hagen, MD, Vonda Wright, MD, Freddie Fu, MD, Johnny Huard, PhD.** Improving Recovery after a Recurrent Hamstring Injury with an Angiotensin II Receptor Blocker Therapy: a Case Report and a Review of Literature (**In preparation**)

Conclusions

This line of work has enabled us to identify three potentially useful compounds that could be utilized to prevent fibrous scar formation and aid in the healing and regeneration of injured skeletal muscle. Suramin is a drug that has been used clinically to treat individuals infected with trypanosomes and worms and is currently being investigated for the treatment of prostate cancer; therefore it could potentially be applied clinically expeditiously. Decorin too has shown great promise; however, it is not currently FDA approved for clinical application and would require more time to apply clinically since it would need to undergo clinical trials. From a basic science standpoint these studies have demonstrated the mechanisms that these two drugs utilize for their beneficial effect on the healing and regeneration of skeletal muscle. They firstly act by inhibiting two components that are major initiators of the fibrosis cascade, TGF-B1 and myostatin. They have also been shown to facilitate regeneration and healing by promoting myoblast and MDSC proliferation and differentiation into myotubes in vitro and myofibers in vivo. One of the pathways that they seem to work on for this enhancement in regeneration appears to be the up- regulation of follistatin. Additional work is currently underway to determine the pharmokinetics of these two promising compounds. Finally we have been exploring the use of the angiotensin II receptor blocker, Losartan, and have

reported the 1st case study that demonstrates this well tolerated drug for the treatment of a patient suffering from recurrent hamstring injury. Additional animal studies are underway to explore the molecular mechanisms involved with the successful use of this drug to treat skeletal muscle injuries besides its' ability to block TGF-B1. We are currently preparing to perform a clinical trial of losartan where the dosing and timing will be evaluated in individuals with similar muscle injuries.

Appendices

Appendix 1: Nozaki M, Li Y, Zhu J, Ambrosio F, Fu FH and Huard J. The use of suramin to improve skeletal muscle healing after contusion injury. Presented as a poster at the Orthopaedic Research Society meeting, Feb. 2007.

Appendix 2: Li Y, Li J, Zhu J, Sun B, Branca M, Tang Y, Foster W, Xiao X, Huard J. Decorin gene transfer promotes muscle cell differentiation and muscle regeneration. Mol Ther 2007 Sep; 15(9):1616-1622.

Appendix 3: Zhu J, Li Y, Shen W, Qiao C, Ambrosio F, Lavasani M, Nozaki M, Branca M, Huard J. Relationships between TGF-β1, myostatin, and decorin: Implications for skeletal muscle fibrosis. J Biol Chem 2007 Aug; 282(35):25852-63.

Appendix 4: Zhu J, Li Y, Huard J. Decorin Interacts with Myostatin Activity-Implications for Skeletal Muscle Healing. The Orthopaedic Research Society (ORS) 53rd Annual Meeting; Feb 12-14, 2007; San Diego, CA

Appendix 5: Zhu J, Li Y, Branca M, Huard J. Follistatin improves skeletal muscle healing after injury. The Orthopaedic Research Society (ORS) 53rd Annual Meeting; Feb 12-14, 2007; San Diego, CA

Appendix 6: Matsuura T, Li Y, Giacobino JP, Fu F, Huard J. Skeletal muscle fiber type conversion during the repair of mouse soleus: Potential implications for muscle healing after injury. J. Orthop Res 2007 Nov; 25(11): 1534-1540**.** Figure featured on front cover as cover art.

Appendix 7: Nozaki M, Li Y, Zhu J, Uehara K, Ambrosio F, Fu F, Huard J. Suramin can enhance the skeletal muscle healing by blocking myostation. The Orthopaedic Research Society (ORS) 54th Annual Meeting; March 2-5, 2008; San Francisco, CA.

Appendix 8: Nozaki M, Li Y, Zhu J, Ambrosio F, Uehara K, Fu F, Huard J**.** Improved muscle healing after contusion injury by the inhibitory effect of Suramin on Myostatin, a negative regulator of muscle growth. Am J Sports Med, 2008 Dec; 36(12):2354-62.

Appendix 9: Bedair H, Karthikeyan T, Quintero AJ, Li Y, Huard J. Angiotensin II Receptor Blockade administered after injury improves muscle regeneration and decreases fibrosis in normal skeletal muscle. Am J Sports Med, 2008 Aug; (36)8: 1548-1555.

Appendix 10: Shen W, Li Y, Zhu J, Schwendener R, Huard J. Interaction between macrophages, TGF-beta 1, and the COX-2 pathway during the inflammatory phase of skeletal healing after injury. J of Cellular Physiology 2008 Feb; 214(2): 405-412.

Appendix 11: Zhu J, Ma J, Lu A, Qiao C, Li J, Li Y, Xiao X, Huard J. Blocking Myostatin by AAV2-Delivered Myostatin Propeptide Improves Muscle Cell Transplantation **.** Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009.

Appendix 12: Uehara K, Nozaki M, Zhu J, Quintero A, Ota S, Fu F, Huard J. Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing in a Dose Dependent Manner. Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009.

Appendix 13: Zhu J, Ma J, Qiao C, Jianbin L, Yong L, Xiao X, Huard J. Blocking Myostatin Improves Muscle Healing Via Enhancement of Angiogenesis. Orthopeadic Research Society; Las Vegas, Nevada; February 22-25, 2009.

Appendix 14: Yuri Chun, MD, James Irrgang, PhD, Tanya Hagen, MD, Vonda Wright, MD, Freddie Fu, MD, Johnny Huard, PhD. Improving Recovery after a Recurrent Hamstring Injury with an Angiotensin II Receptor Blocker Therapy: a Case Report and a Review of Literature (**In preparation**)

Project # 2
Progress Report
Improving muscle healing through digestion of scar tissue via MMP-1
(Yong Li)

Introduction:

Muscle injuries, which occur most frequently during sports activity, military training and battle; present a challenging problem in traumatology. After injury, damaged muscle fibers undergo a natural process of necrosis and the resulting dead tissue is removed by infiltrating lymphocytes. Meanwhile, locally released growth factors stimulate muscle regeneration by activating satellite cells. Unfortunately, the process of muscle regeneration is often incomplete from overgrowth of the extracellular matrix (ECM) leading to significant local fibrosis (i.e., fibrous scar formation). This scar tissue impedes the formation of normal muscle fibers in the injured muscle, resulting in incomplete functional recovery and a propensity for re-injury. We have begun to study the mechanism behind the fibrosis that occurs in injured skeletal muscle. Our previous studies have demonstrated that myogenic cells (including muscle-derived stem cells [MDSCs]) and regenerating myofibers in lacerated muscle can differentiate into fibrotic cells, and that transforming growth factor (TGF)-beta1 is a major stimulator of this differentiation. Using different animal models of muscle injury, we have investigated biological approaches by which to prevent fibrosis and thereby improve muscle healing. However, it often is not possible to treat injured muscles before the initiation of fibrosis—most patients with muscle injuries seek treatment only after the onset of fibrous scar formation, and the concomitant pain and functional deficits it produces. Moreover, chronic diseases (e.g., Duchenne muscular dystrophy [DMD]) generally present significant amounts of fibrous scar tissue within the patients' muscles. Because prevention of fibrosis is infeasible in many cases, the development of a novel therapeutic approach by which to digest existing fibrous scar tissue and improve muscle healing would be very significant.

Matrix metalloproteinase type-1 (MMP1), a naturally occurring collagen-digesting enzyme, has shown great capacity for digesting fibrous scar formations in various tissues. Additionally, MMP1 is also able to increase cell migration in many tissues. We believe that MMP1 is able to facilitate the healing of injured muscle by digesting fibrous scar tissue and improving the local environment in which muscle regeneration occurs. Results obtained from this project may lead to the development of gene therapy applications that eliminate scar tissue within skeletal muscle. Such applications could drastically improve the regeneration of muscles damaged by trauma or by chronic muscle diseases, such as Duchenne and Becker muscular dystrophies.

In the past two years, we have completed most of the proposed experiments. Through our work on **Objective #1**, we have determined that MMP1 increased muscle cell migration and differentiation/fusion capacity in vitro. In vivo, we also determined that MMP1 could improve muscle healing through increasing muscle cell migration, differentiation and regeneration (**Objective #2**). Finally, we have discovered that MMP1 gene transfer enhanced muscle cell migration and differentiation in vitro and in vivo (**Objective #3**).

Body:

Technical Objective #1: To assess the effect of MMP1 on muscle cells *in vitro*.

This proposed experiment has been completed in the past two years. Results from this objective indicate that MMP1 is able to inhibit fibroblast growth and collagen deposition as well as enhance myoblasts migration and fusion/differentiation of these cells *in vitro*. The completed experiment has carried out two publications in the *Journal of Applied Physiology* [2007;102(6):2338-2345] and *American Journal of Pathology* [2009;174(2);541-549].

Technical Objective #2: To assess the effects of MMP1 on skeletal muscle *in vivo*.

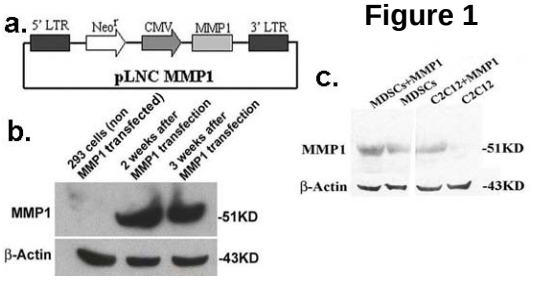
In this objective we observed the effects of MMP1 on skeletal muscle healing in animal models *(in vivo*). We have finished most of this proposed experiment. By using a reproducible muscle injury model in mice that induced significant fibrous scar formation, hindered myofiber regeneration and slowed muscle healing, we have determined that MMP1 can digest the existing fibrous scar and promote muscle healing in the injured skeletal muscles. This proposed experiment has been completed in the past year, and this study has been published by the *Journal of Applied Physiology* 2007;102(6):2338-2345 (Please see attached copy of manuscript). In addition, to observe the effect of MMP1 on muscle cell migration and differentiation in vivo, we have selected MDX mice, a mouse model for Duchenne muscular dystrophy [DMD], to investigate if MMP1 could enhance muscle cell migration and differentiation in vivo. Our results have substantiated this point and showed that MMP1 could enhance muscle cell fusion as well as migration in dystrophic muscle. This research have been published by *American Journal of Pathology (*2009;174(2);541-549*).* (Please see attached copy of manuscript).

Technical Objective #3: To assess the use of MMP1–based gene therapy to digest fibrous scar tissue within injured skeletal muscle.

The primary aim of this proposal is to investigate whether MMP1 gene transduction in implantable myoblasts can extend MMP1 function as a means of digesting fibrous scar tissue and promoting muscle cell migration, fusion and regeneration within injured skeletal muscle. MMP1 can degrade collagens, but undergoes self–degradation, which results in a short biological half-life. As a result, the fibrous scar tissue has the potential to reform following MMP1 degradation. To extend the function of MMP1, we have constructed a retrovirus vector encoding the MMP1 gene. The updated results as following:

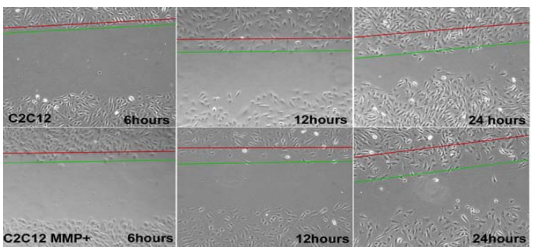
1. MMP1 plasmid constructs: To extend the function of MMP1, we have constructed a retrovirus vector encoding the MMP1 gene. The Bgl II (blunt ended)/Sal I fragment of pCllase I (ATCC, Rockville, MD), including all coding regions of the human MMP1 gene, was cloned into the Not I (blunt ended)/Xho I site of pLNCX2 (Retroviral Vector, CLONETECH) to generate pLNC MMP1 (***Fig. 1a***). Phoenix 293 cells were transfected (ATCC, Rockville, MD) with pLNC MMP1 using the liposome technique (DOTAP, Boehringer Mannheim), and G418 medium was used to select for the transfected cells. Western blot analysis was used to verify the selected clone cells' expression of MMP1 (***Fig. 1b***). The supernatant from the culture containing the cloned 293 cells was collected to evaluate the titer and transfer to myoblasts (C2C12) and MDSCs. Both transferred C2C12 and MDSC cultures expressed high levels of MMP1 (***Fig. 1c***).

Figure 1



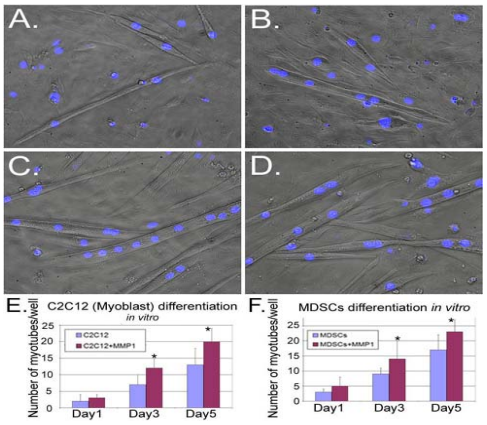
2. MMP-1 gene transduction stimulated myoblasts to increase migration. MMP1 gene transferred C2C12 cells and control C2C12 cells were cultured in medium containing Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA), 10% fetal bovine serum, 10% horse serum, 0.5% chicken embryo extract, and 1% penicillin/streptomycin (P/S) at 37oC in a 5% CO2 atmosphere in 12 well plates. Cells were then placed in serum-free DMEM supplemented with 1% P/S alone. An artificial wound was created by disrupting the monolayer with a sterile plastic pipette tip. Cells were incubated for 6, 12 and 24 hours to allow for migration back into the wound area. Cells were then fixed in cold methanol, washed with PBS, and then stained with DAPI to help visualize migration distance. We observed that MMP-1 gene transduction (*bottom row*, ***Fig. 2***) promoted C2C12 cell migration into an artificially created wound faster than control C2C12 (*top row*, ***Fig. 2***).

Figure 2



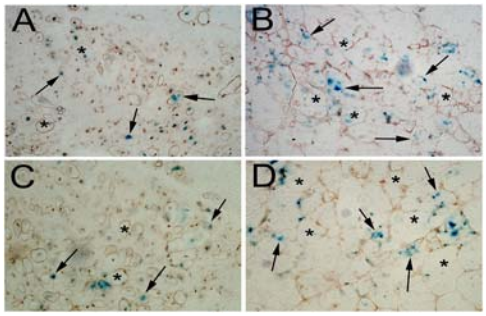
3. Gene transfer of MMP1 enhances myoblast differentiation *in vitro*. We investigated whether MMP1 gene transduction has any effect on myogenic cell differentiation. MMP1 cloned myoblasts (C2C12), MDSCs and their control non-gene-transferred cells were separately plated into 12-well-plates and cultured with differentiation medium (2% H.S.) for up to 5 days. Results indicate that the MMP1 gene-transferred myogenic cells resulted in increased numbers of fused myotubes compared to control cells (***Fig. 3***). Myoblasts (C2C12) (***Fig. 3A***) and MDSCs (***Fig. 3B***) fused into myotubes in differentiation medium at their usual levels, however, both MMP1 gene-transferred myoblasts (***Fig. 3C, E***) and MDSCs (***Fig. 3D, F***) increased their differentiation capacity (*P<0.05). It is unclear what mechanism underlies MMP1's up-regulation of the differentiation of myogenic cells, however, MMP1 down-regulation of fibronectin could be one of the culprits 9. It is also possible that MMP1 can directly affect myogenic cell fusion 10. Our further studies will investigate the relationship between MMP1 expression, the level of fibronectin secretion and myogenic cell differentiation, as well as the underlying mechanisms involved.

Figure 3



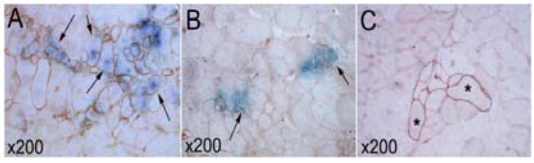
4. MMP1 gene therapy improves myoblast transplantation. To investigate whether these MMP1-expressing cells are able to produce a better muscle graft in the skeletal muscle of MDX/SCID (a dystrophic/immunodeficient mouse model) mice, we used a retrovirus vector to transfer the *LacZ* marker gene into genetically altered C2C12 cells that expressed MMP1 as well as control C2C12 cells that did not express MMP1. These cells were then separately transplanted into the skeletal muscle of *MDX*/SCID mice. Histological analysis was performed after cell transplantation, whereupon we detected both LacZ-positive (blue; see arrows in ***Fig. 4A-D***) and dystrophin-positive (brown; see asterisks; ***Fig. 4A-D***) myofibers in the host muscle at 2 weeks (***Fig. 4A, B***) and 4 weeks (***Fig. 4C, D***) after cell transplantation. However, we discovered that the MMP1-expressing C2C12 fuse/differentiate into better muscle grafts (***Fig. 4B, D***) after transplantation into the skeletal muscle of *MDX* mice than the control C2C12-transplanted groups (***Fig. 4A, C***) at both 2 and 4 weeks after cell transplantation. In future studies, we will measure the number and the diameter of LacZ-positive and dystrophin-positive myofibers from the skeletal muscle of control and MMP1-expressing cell therapy groups.

Figure 4



5. MMP1 gene transduction increases myoblast migration *in vivo*. We selected both genetically engineered MMP1 expressing C2C12 (myoblasts) and C2C12 control cells as donor cells. Both cells were marked with the LacZ gene as a track marker (see above). Equal quantities of MMP1-gene-transferred C2C12 cells and control C2C12 cells (1x105 cells) were separately injected systemically into the tail veins of MDX/SCID mice. At various time-points (1, 2, and 3 weeks) after injection, the mice were sacrificed, and the muscle tissues including skeletal muscles, heart, and diaphragm muscles were harvested for histological analysis. In the MMP1 gene-transfected C2C12 injected mice, we detected some LacZ positive myofibers within the skeletal muscles at 2 weeks after injection (***Fig. 5A***). By immunohistochemistry of ABC staining (ABC kit, Vector Laboratory, Inc., CA), we discovered that the location of the LacZ positive myofibers matched the location of the dystrophin positive myofibers in these muscles (arrows, ***Fig. 5A***). However, there were few LacZ and dystrophin positive myofibers detected within the skeletal muscle of the control (non-MMP1 treated) C2C12 injected

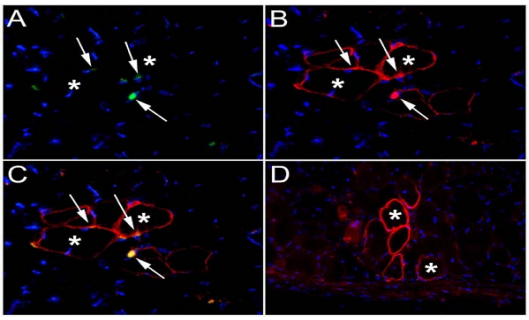
Figure 5



mice (*arrows*, ***Fig. 5B***); furthermore, some of the dystrophin positive, LacZ negative myofibers (*asterisks*, ***Fig. 5C***) could be reverted myofibers that were located in the skeletal muscle of MDX mice. Results from this pilot study could support the potentially beneficial effect of MMP1 on myogenic cell migration. Western blot analysis has provided evidence that MDCSs alone can express a low level of MMP1 compared to the C2C12 myoblasts (***Fig. 1C***). In fact, our previous study also observed MDSCs displaying better migratory capacity in the *in vivo* animal model 11,12. The migratory ability of MDSCs may be due to the low-level expression of MMP1.

6. Myoblasts genetically engineered to express MMP1 display a strong migration capacity following systemic delivery into *MDX* mice. To investigate whether the systemic delivery of MMP1-expressing myoblasts could migrate into the diaphragm of *MDX* mice, we have performed immunohistochemistry to examine co-localization staining of the LacZ marker and dystrophin genes in the host diaphragm. Results show that neither LacZ-positive (b-galactosidase) nor dystrophin-positive myofibers were found in the diaphragm muscle following systemic delivery of the control C2C12 into *MDX* mice. We did, however, discover LacZ-positive myofibers (*green, arrows;* ***Fig. 6A, C***) co-expressed with dystrophin myofibers (*red, asterisks*; ***Fig. 6B, C***) within the diaphragm of *MDX* mice after systemic injection of C2C12 that were genetically engineered to express MMP1. We also detected some myofibers that expressed dystrophin within the intercostal muscles of the *MDX* mice that received systemic delivery of our MMP1-expressing C2C12 (*red, asterisks*; ***Fig. 6D***). These results support the notion that MMP1 is able to increase myoblast migration and may help spread donor cells to all types of muscles after systemic delivery.

Figure 6



Future plans: We have successfully completed **Objective #1 & #2**. For **Objective #3**, we have finished a majority of the proposed experiment. We will focus on preparing manuscripts and one other minor experiment. Based on our results, the new proposal is under discussion with my research team. We expect to investigate the mechanism behind MMP1 in the muscle healing process in the forthcoming project (if funded), by examining how the MMP1 application or MMP1 gene therapy relates to muscle cell precursors, e.g. satellite cells and stem cells populations. More importantly, we have discovered a potential relationship between MMP1 application and angiogenesis or reinnervation, both of which are key aspects of functional recovery in injured skeletal muscle.

Key Research Accomplishments:

1. Discovery of the effect of MMP1 on muscle cells, resulting in increased migration and differentiation of myogenic cells *in vitro*.
2. Discovery of the effect of MMP1 on scarring skeletal muscle, namely, the digestion of fibrous scar tissue, and improved muscle healing *in vivo*.
3. Discover of the effect of MMP1 on muscle cell migrations and fusion in skeletal muscle of mice *in vivo*.
4. Discovery of MMP1 gene transfer to extend its half-life, which prolongs MMP1 function during its application both *in vitro and in vivo*.

Reportable outcomes:

Two manuscripts have been published:

1. Bedair H, Liu TT, Kaar J, Shown B, Russell A, Huard J, Li Y***,** Matrix Metalloproteinase (MMP) Therapy Improves Muscle Healing. ***J Applied Physiology*** 2007 Jun;102(6):2338-45.

2. Wang W, Pan HY, Murray K, Jefferson M, Li Y. MMP1 promotes muscle cells migration and differentiation. ***American J Pathology*** 2009, 174 (2); 541-549.

We have prepared another manuscript to summarize our results from **Objective #3** to be entitled, "MMP1 gene therapy to enhance muscle cell migration and differentiation".

Six Abstracts have been presented at five different national and international conferences and have won two awards in past years: The Award for Outstanding Research in Biochemistry/Cell Biology and the prestigious Award for Overall Excellence in Research at the 2006 AMA-MSS National Research Poster Competition.

Besides this funded study, we have been invited to write MMP related ***Three Reviews*** as following journals:

1. Mu XD, Wang W, Li Y. Myoblast transplantation and fibrosis prevention in diseased muscle (invited review). ***US Musculoskeletal Review,*** (2009; 4:1)

2. Bellayr I, Li Y. Biochemical insights into the role of matrix metalloproteinases in the regeneration: challenges and recent developments. Future Medicinal Chemistry ***(invited review) (2009)***

3. ***Chen XP, Li Y. Role of*** matrix metalloproteinases in skeletal muscle: migration, differentiation, and regeneration. ***Cell Adhesion & Migration*** (invited, 2009)

Conclusion:

In vitro, we have seen that MMP1 can enhance muscle cell migration and differentiation. The migration–related proteins are also up–regulated within MMP1–treated muscle cells. *In vivo*, the fibrous scar tissues that form within traumatically injured skeletal muscle could limit transplanted myogenic cell migration, fusion, and regeneration, thus slow the overall muscle healing process. However, muscle healing was greatly improved following MMP1 treatment within these scarred skeletal muscles. We also discovered that the use of MMP1 gene–transferred myoblasts resulted in an increased differentiation capacity *in vitro* and enhanced muscle cell migration *in vivo*.

Appendix and Reference:

The manuscript entitled "Matrix Metalloproteinase Therapy Improves Muscle Healing" has been published on the ***Journal of Applied Physiology*** 2007;102(6):2338-2345 resulted from **Objective #2**, The manuscript entitled "MMP1 enhances muscle cell migration and differentiation" has been published on the ***American J Pathology*** 2009, 174 (2); 541-549 resulted from **Objective #1 & #2**, and Six abstracts which are attached in the **Appendix**. The majority of relevant references may also be found in these manuscripts. Three review articles will be showed public soon.

Project # 3
Final Report
Repairing injured skeletal muscle through myogenic endothelial cells
(Bruno Peault)

INTRODUCTION

Upon muscle injury, satellite cells, the professional myogenic progenitors present in skeletal muscle, can divide, differentiate and fuse to generate myofibers [1, 2]. Injection of myoblasts has been attempted to repair both skeletal and cardiac muscles in animals and humans [3-5] and was to some extent successful but severe limitations were also encountered after myoblast implantation in both tissues[3, 6, 7,]. Muscle regeneration might be improved by transplanting stem cells, in some instances after therapeutic gene transfer, instead of later myogenic progenitors [8-12].

As described in other sections of this report, we have previously isolated, based on their slow adherence in culture, early myogenic progenitor cells, named muscle derived-stem cells (MDSC), that more efficiently regenerate both skeletal and cardiac muscles than satellite cells [9, 13]. MDSC were isolated retrospectively from cultured skeletal muscle; therefore their origin, identity and anatomic location in the native muscle are still unknown. The expression by MDSC of the myogenic cell markers desmin and MyoD, in addition to CD34 and Sca-1 and absence of c-kit and CD45, distinguishes these cells from other known adult stem cells such a MSC and MAPC.

We have, in the present study, investigated the identity and anatomical location of muscle-derived stem cells in human skeletal muscle. Our conclusion is that a previously unexplored developmental relationship exists between endothelial and myogenic cells. Indeed, a subset of MDSC express endothelial cell markers, and MDSC spontaneously differentiate into endothelial cells, and/or promote angiogenesis, probably *via* VEGF secretion, following implantation in skeletal and cardiac muscles [9, 13].

Besides regular satellite cells and endothelial cells, we have identified and characterized a novel population of *myo-endothelial* cells that co-express myogenic and endothelial cell markers. When injected into the injured skeletal muscles of immunodeficient mice, FACS-sorted cells co-expressing myogenic and endothelial cell markers (CD56+CD34+CD144+) regenerated skeletal muscle fibers much more efficiently than conventional CD56+ myogenic cells. Myoendothelial cells clonally expanded from single sorted cells differentiated into myogenic, chondrogenic and osteogenic cells under appropriate culture conditions.

KEY RESEARCH ACCOMPLISHMENTS

We have achieved the molecular identification and sorting of human myo-endothelial cells. Human skeletal muscle sections were immunostained with antibodies directed against myogenic and endothelial cell antigens. A subset of satellite cells (Pax7+ or CD56+) co-express endothelial cell antigens: VE-cadherin (CD144), vWF, the UEA-1 receptor and CD34. The percentages of Pax7+ cells also expressing these endothelial cell antigens were, respectively, 8.9%, 9.5%, 9.5% and 9.8%, as counted on at least 100 satellite cells in five independent experiments. Co-expression of these molecules by individual cells was ascertained by confocal microscopy. These results suggested the existence within adult human skeletal muscle of a rare subset of cells that co-express myogenic and endothelial cell markers. Adult skeletal muscle samples were then analyzed by flow cytometry after enzymatic dissociation. CD45- viable cells were gated and further separated into CD56+ myogenic cells and CD56- non-myogenic cells. As expected, the latter subset of non-myogenic cells contained the bulk of endothelial cells (CD34+CD144+). However, CD56+ cells also contained a very minor subset of cells expressing CD34 and CD144, thus confirming the existence of cells co-expressing markers of the myogenic and vascular endothelial cell lineages. Using the same immunostaining conditions, we then proceeded to sort the above-described cell subsets by FACS. The mean numbers of viable sorted cells which were recovered per experiment were $4.8 \pm 1.3 \times 10^4$ CD56+CD34-CD144- myogenic cells, $7.7 \pm 2.6 \times 10^4$ CD56-CD34+CD144+ endothelial cells and $1.5 \pm 0.8 \times 10^4$ CD56+CD34+CD144+ myoendothelial cells.

Purities of these three sorted cell populations were, respectively, 93.8 ± 1.0 %, 92.2 ± 1.1 % and 93.5 ± 1.8% [14-16]

We have regenerated skeletal mucle with human myoendothelial cells Myoendothelial, endothelial and satellite cells sorted from adult skeletal muscle were injected intramuscularly, in multiple distinct experiments, into SCID mouse skeletal muscles that had been injured by cardiotoxin. After 10 days, mice were sacrificed and skeletal muscles were analyzed by immunohistochemistry using an antibody directed to human spectrin. All three cell categories tested regenerated spectrin-expressing muscle fibers but quantitative analysis revealed that the regenerative potential of myoendothelial cells is by far the highest. Indeed, 1000 myoendothelial cells generated on average 89 myofibers, as compared with 9 and 5 myofibers produced when the same number of cells expressing exclusively endothelial or myogenic cell markers was injected, respectively. These results support our hypothesis that muscle vascular endothelial cells and a novel subset of cells with an overlapping phenotype between myogenic and endothelial cells are endowed with high muscle regeneration potential[14-16]

We have cultured myoendothelial cells over the long term, and shown that these proliferate faster, and survive better under oxidative stress than myogenic cells and endothelial cells Endothelial, myoendothelial and satellite cells were cultured independently in the medium used for MDSC culture[9] for 5 to 6 weeks. All 3 subsets of long-term cultured cells remained capable of differentiating into myotubes in vitro. Cultured cells injected into SCID mouse muscles damaged with cardiotoxin also retained their ability to regenerate myofibers, and myoendothelial cells remained the most efficient myogenic progenitors in these assays.

Myoendothelial cells proliferate significantly faster than the two other cell groups. Having previously observed that the high regenerative capacity of mouse MDSC in skeletal and cardiac muscles is related to their resistance to oxidative stress [13], we treated the three populations under study with hydrogen peroxide in culture. Myoendothelial cells are the most resistant to oxidative stress as indicated by lower levels of cell death under these conditions. [14-16]

We have shown that myoendothelial cells are, at the clonal level, multi-lineage mesodermal stem cells We investigated the multipotency of myoendothelial cells, isolated from adult human skeletal muscle, at the single-cell level. Myoendothelial cell clones express markers of myogenic, endothelial, perivascular and mesenchymal stem cells (MSCs). These clonal cells can be differentiated into endothelial cells, smooth muscle cells, pericytes, adipocytes, chondrocytes and osteoblasts *in vitro* and participated in vessel, bone and cartilage regeneration *in vivo*. We therefore demonstrate that human myoendothelial cells possess some fundamental properties of stem cells.[17]

REPORTABLE OUTCOMES

- Peault, B. et al. Stem and Progenitor Cells in Skeletal Muscle Development, Maintenance, and Therapy. *Mol Ther* (2007)
- Zheng, B., Cao, B., Crisan, M., Sun, B., Li, G.H., Logar, A., Yap, S., Pollett, J.B., Drowley, L., Cassino, T., Gharaibeh, B., Deasy, B., Huard, J., Péault, B. Prospective identification of myogenic endothelial cells in human skeletal muscle. *Nature Biotechnology*, 25(9):1025-1034 (2007
- Crisan, M., Deasy, B., Gavina, M., Zheng, B., Huard, J., Lazzari, L., Péault, B. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. *Methods In Cell Biology*, 86:295-309. (2008)
- Crisan, M., Zheng, B., Sun, B., Yap, S., Logar, A., Huard, J., Giacobino, J.P., Casteilla, L., Péault, B. Purification and culture of human blood vessel-associated progenitor cells. Current Methods in Stem Cell Biology. In Press.
- Zheng, B., Li,G., Deasy, B., Pollett. J., Sun, B, Drowley, L., Gharabeh, B., Usas, A., Logar, A, Peault, B., and Huard, J. The myoendothelial cell: a novel stem cell from adult human skeletal muscle. *In preparation*

Although the present project has been focused on the regeneration of *skeletal* muscle, we have also documented the strong potential of these cells to regenerate the myocardium and improve cardiac function post-infarction:

- Okada, M., Payne, T., Zheng, B., Oshima, H., Momoi, N., Tobita, K., Keller, B.B., Phillippi, J., Péault B., Huard, J. Improved cellular cardiomyoplasty via human myogenic-endothelial cells identification of a progenitor cell population from human skeletal muscle that is superior to committed skeletal myoblasts for cardiac cell therapy. *Journal of the American College of Cardiology*. (2008)

CONCLUSIONS

The experiments performed in this project have provided evidence for the existence of a strong myogenic potential in cells related to the endothelial cell lineage in the normal, human adult skeletal muscle, and therefore suggest a developmental relationship between vascular cells and myogenic cells. We have indeed confirmed the existence of a novel population of cells present between human muscle fibers that co-express the nuclear Pax7 myogenesis-specific transcription factor and surface antigens that typify endothelial cells: VE-cadherin (CD144) and vWF.

The leading conclusion of the present work is that human muscle-derived myoendothelial cells can regenerate skeletal muscle, quantitatively more efficiently than regular myogenic (satellite) cells. The higher regeneration capacity exhibited by myoendothelial cells is due in part to better resistance to oxidative stress, a likely environmental condition within the injured skeletal muscle. These data suggest the existence of a novel hierarchy within human adult skeletal muscle along which vascular endothelial cells give rise to early myogenic stem cells, which in turn replenish the satellite cell population.

These novel myogenic progenitors are amenable to biotechnological processing; these cells can be sorted to homogeneity by flow cytometry from skeletal muscle, then cultured clonally on the long term, with no major loss of developmental potential, in basic medium, where they proliferate rapidly but do not become tumorigenic. Therefore, the transplantation of such autologous endothelium-related progenitors could be envisioned shortly as a therapy of skeletal muscle diseases and injuries.

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15. Crisan, M., Deasy, B., Gavina, M., Zheng, B., Huard, J., Lazzari, L., Péault, B. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. *Methods In Cell Biology*, 86:295-309. (2008)
16. Crisan, M., Zheng, B., Sun, B., Yap, S., Logar, A., Huard, J., Giacobino, J.P., Casteilla, L., Péault, B. Purification and culture of human blood vessel-associated progenitor cells. Current Methods in Stem Cell Biology. In Press.
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Project # 4
Progress Report
Cell therapy for muscle regeneration advances via interdisciplinary-driven regenerative medicine (iDREAM)
(Bridget Deasy)

Key Research Accomplishments

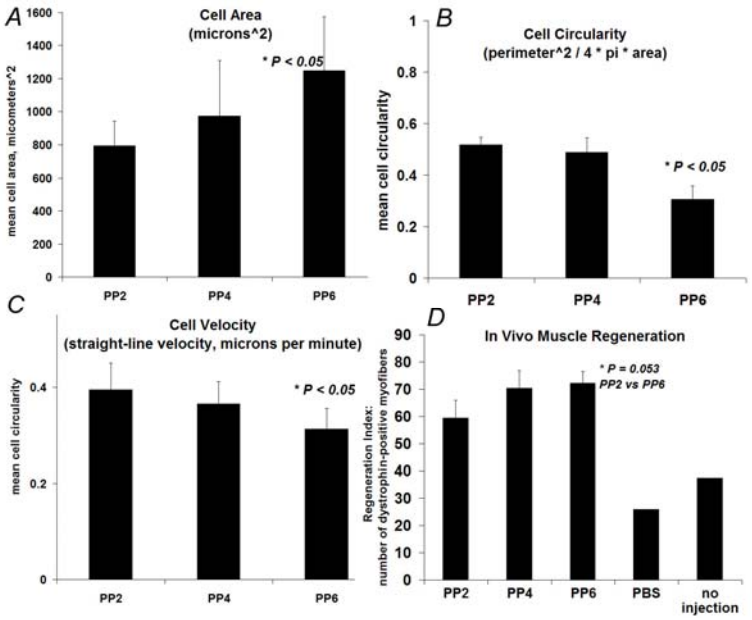
1. **Technical Objective #1:** Use preplate isolation technique and a bioinformatic cell culture system to screen adult human stem cell candidates for their muscle regeneration potential.
2. **Technical Objective #2:** Test culture conditions for extensive and long-term expansion of human myogenic cells to preserve the cells' phenotype and regeneration efficiency.

We have completed these aims. First we have identified that the most potent human muscle stem cell candidate can be isolated from slowly adhering fractions of the cell suspension which derives from the muscle biopsy. To identify these cells we first examined the cells via live cell imaging, and second we examined the cells in an in vivo model of muscular dystrophy.

Through a collaboration with Cook MyoSite Inc (Pittsburgh, PA), we received samples of human muscle cells. These cells were isolated based on their variable adherence, using a method similar to the preplate technique which was performed with mouse myogenic cells. It has been previously shown that the preplate isolation technique can be used to isolate stem cells from the skeletal muscle of mouse [32] and rat [75], while direct defined FACS-sorting has been performed using tissue from mouse [32], rat [75] and human sources[18]. Usually, myogenic precursors with characteristics of myoblasts or satellite cells are found within the early preplates (PP1–3), whereas cells isolated from the later preplates (PP5-6) have stem cell characteristics[1]. We term the cells PP2, PP4 and PP6-- PP2 adhere prior to PP4 which adhere prior to PP6.

These results provide examples that illustrate a relation between in vitro and in vivo characteristics. Human MDCs were analyzed for the presence of the cell surface cluster of differentiation markers CD34, CD56, CD144, and CD146 by flow cytometry. For live cell imaging analysis 3 separate hMDCs preplate populations were examined – PP2, PP4, and PP6. By the preplate method, PP2 corresponds to myoblasts and PP6 corresponds to the muscle-derived stem cell candidate[1,77,79]. The cells were imaged using the automated cell imaging system[81]. Three separate visible image sequences (20x) were captured at 10 minute intervals for each well over a culture time of 3 days. Image sequences were imported to ImageJ for user interactive analysis. Measurements included 17 parameters including area, centroid, center of mass, perimeter, circularity, skewness, and best fit ellipse. Sequences were divided into 18 hour increments with 10 separate cells outlined and measured over 30 minutes at each time point. Results from ImageJ measurement were imported into a predesigned worksheet for calculation and analysis. Cell motility was described as centroid velocity, dividing the change in centroid position by the scan time interval. Single cell measurements were averaged over each time point and image sequence yielding a morphological description of each well for the culture period. The mean area for PP2 (792±151 um^2) was

Figure 1



significantly smaller (P=0.022, type-2, 2-tailed) than PP6 (1248±327 um^2, **Fig 1A**). Furthermore, PP2 velocity (0.395±0.056 um/min) was significantly greater (P=0.031) than PP6 (0.312±0.044 um/min, **Fig 1B**). Finally, examination of circularity (a measure of cell roundness) showed PP2 (0.517±0.028) and PP4 (0.487±0.058) were significantly different from PP6 (0.307±0.052, P<0.01, **Fig 1C**). Flow cytometry showed all PPs were negative for CD34 & CD144, with decreasing expression for CD146 & CD56 over time in culture. In sum, the results show that PP2 cells are significantly smaller and more motile with greater circularity than PP6, two populations previously resolved only by adherence rates. The LCI approach shows the ability to resolve differences in cell populations based on a number of behavioral measures only a few of which are presented here. Moreover subsequent in vivo experiments showed that this in vitro difference correlates with an in vivo difference in performance (**Fig 1D**).

We have also expanded these cells under various cell culture conditions and we confirm that the phenotype of the cells is altered through standard culture conditions (**Fig 2**). We found that cell lose expression of the myogenic marker CD56 and the progenitor marker CD146 as they are expanded (**Fig 3**) as the are expanded.

It is worth noting that whereas we have expanded mouse MDSC populations to beyond 200 population doublings (PDs), our results to date show that human MDSC populations will reach senescence much sooner than mouse MDSCs when grown in the same growth medium of 20% serum Dulbecco's Modified Eagle Medium (DMEM). **Figure 2A** depicts these differences in the proliferation kinetics of human and mouse MDSCs (in 20% DMEM, 10^9 mouse MDSCs vs. 10^{12} human MDSCs, at 7 weeks, n=1). These results underscore the need to more extensively study growth factor-expansion methods for human stem cells. Using commercially available, chemically defined media, we were able to stimulate human muscle–derived stem cell expansion in the *in vitro* expansion assay (**Figure 2**). We have observed that other human muscle cells-myoendothelial cells-have exhibited increased expansion when cultured in defined media such as endothelial growth medium 2 (EGM2, Clonetics) or skeletal basal medium (SKBM, Clonetics) ([56], **Figure 2B**). Expansion of FACS-isolated human myogenic progenitors was examined using 3 different culture media (20% serum, EGM2 and SKGM [56]). Three

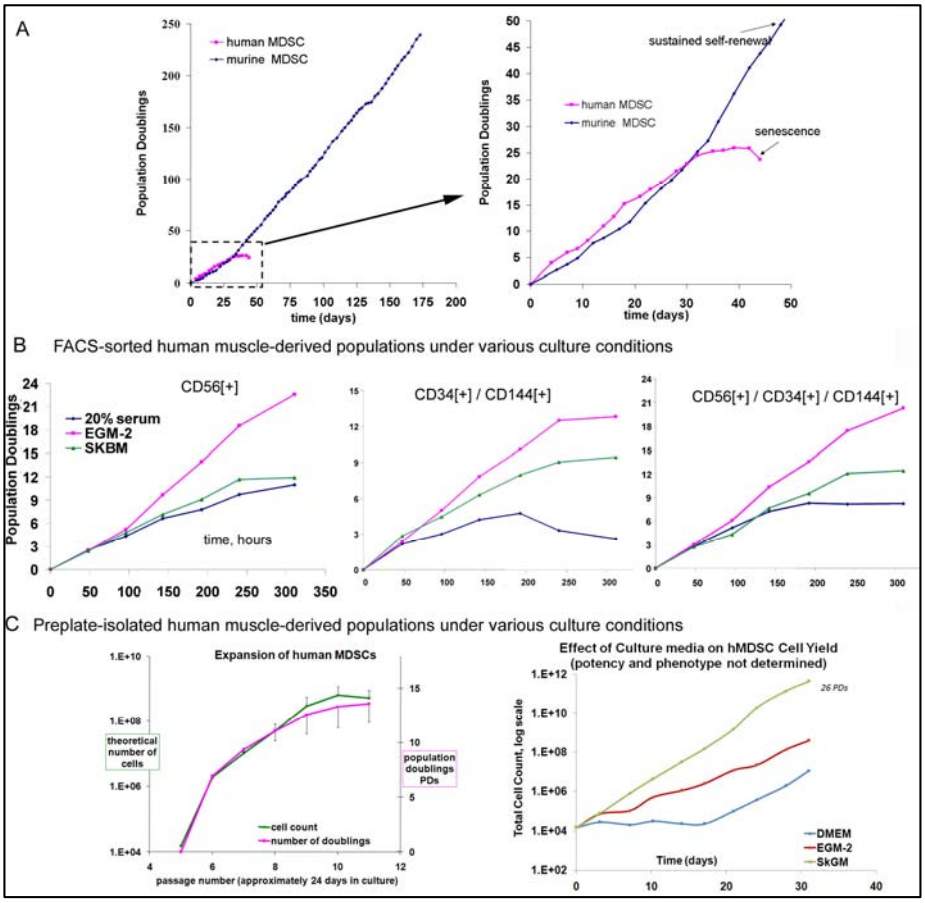


Figure 2. Human MDSC expansion. (A) hMDSC have slower proliferation rates as compared to mouse MDSC *Human myogenic cells were cultured using the same techniques used for mouse MDSCs; yet, a striking difference in their expansion potential was observed.* ***(B)*** *Expansion of FACS-isolated human myogenic progenitors was examined using various culture media (20% serum, EGM2 and SKGM). Medium supplemented with endothelial growth factor (EGM2) stimulated the greatest amount of expansion for all hMDCs. The results of panel (B) were published in Zheng et al 2007 Nat Biotech.* ***(C)*** *While hMDSC show promise for clinical treatment of urinary incontinence much work needs to be performed to expand these cells to higher cell doses to reach a much larger set of tissues to be effective for muscular dystrophies.*

purified populations were examined – CD56[+] cells, CD34[+]CD144[+] cells and CD56[+] CD34[+] CD144[+] cells. All three purified human populations demonstrated the highest degree of expansion with EGM-2 media **(Figure 2B).**

The results shown in **Figure 2B** were recently included in a report by Zheng et al, in which the authors suggest that the MDSCs may have their developmental origins as myoendothelial cells[56,57]. Subsequently we performed another examination of the effect of 3 different culture media on the preplate derived human MDSC populations. In this case, we observed the greatest expansion potential with SKGM (**Figure 2**), and not EGM2 which favored human myoendothelial cells sorted by FACS. While this may suggest that the human myoendothelial cells and the human preplate MDSCs are unique populations, those experiments have not been performed side-by-side, and it is not our goal to compare these populations. Rather, these findings show that optimization of cell expansion is cell-line specific and the human preplate cells can be expanded to high numbers using SKGM as compared to EGM2 (**Figure 2**). We examined phenotype during the course of cell expansion. All 3 preplate fractions were initially negative for CD34 and CD144 (data not shown). The PP2, PP4, and PP6 fractions expressed significantly different amounts of the myogenic marker desmin (**Figure 3A**) with the PP2 (fast adhering) fraction containing the highest percentage of positive cells ($p < 0.05$ as compared to the slow adhering or PP6 fraction). We also observed variable levels of CD56 and CD146, and these markers decreased with cell passage. **Figure 3B** shows the changes in CD56 which occur as cells are cultured. The fast-adhering (PP2) fraction, and moderately adhering (PP4) fraction show a decrease in CD56 expression through the course of 3 cell passages (passage 7 through passage 9, **Figure 3B**). The slow (PP6) fraction maintains a low level of CD56 through the course of these passages. We have also examined the PP6 / hMDSCs fraction for expression of CD90, CD105 and CD73, which are expressed by mesenchymal stem cells. The populations are >90% positive for these markers (flow cytometry and PCR results, data not shown). These results and others suggest that hMDSCs may be obtained from the PP6 fraction which appears to be distinct from PP2.

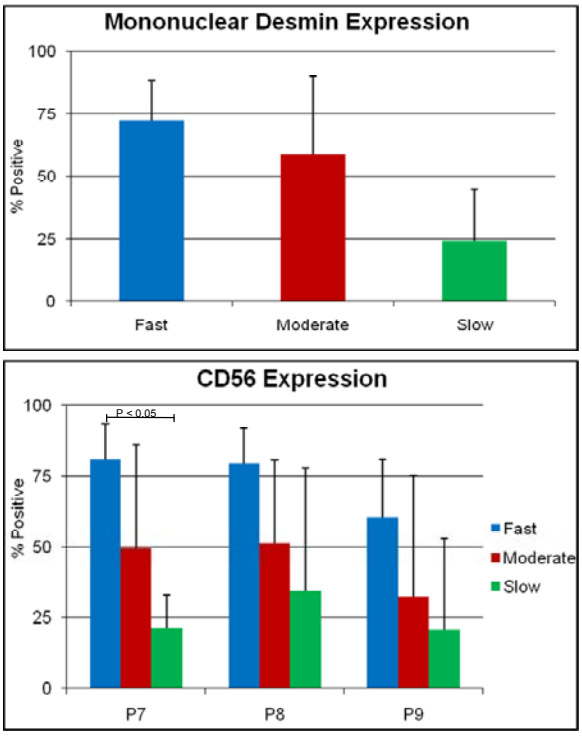


Figure 3. *Human preplate populations. PP2=Fast adhering fraction, PP4=moderate rate of adherence, PP6=slow adherence rate during the preplate isolation process. These populations differ in their expression of* ***(A)*** *desmin (via immunocytochemistry) and* ***(B)*** *CD56 (via flow cytometry). We also illustrate in (B) that the expression of this marker changes with cell expansion (passage 7-passage 9 are shown).*

Supplemental Objectives

Technical Objective #1: Use a bioinformatic LCI culture system to test the effect of temperature on the processing of MDSC from skeletal muscle biopsy. Here we will consider the various temperature cycles that the biopsy may undergo prior to stem cell isolation. We will examine differences in percent yield based on the length of time that a biopsy is at 4C storage. We will examine the phenotypes of MDSCs from various isolations using the LCI tools.

Progress: The instrumentation for these studies is now in place and we have initiated studies of the effects of temperature on stem cell viability. The delivery of the imaging system for these experiments was delivered on Jan 14, 2009 and calibration was complete on Jan 30, 2009. Therefore, it was necessary for us to request a no-cost extension on these objectives. We have performed initial experiments to examine cell viability at lower

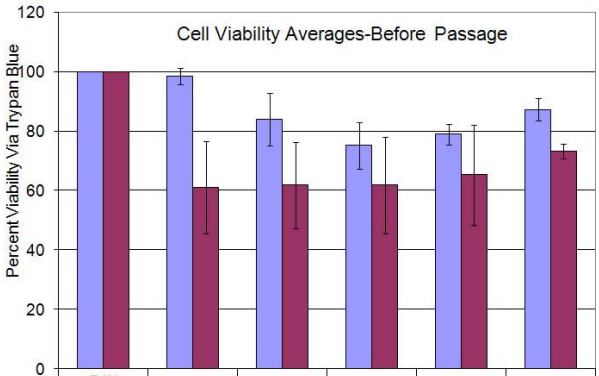
temperatures and to date we confirmed viability and cell proliferation, however, the complete analysis of the effect of temperature is still ongoing.

Technical Objective #2: To test the effect of various cyroprotectant agents and procedures on the stem cell phenotype of human MDSC. In this objective, we will obtain unique comprehensive phenotype profiles of muscle-derived stem cell populations after various preservation protocols. We will also examine various thawing procedures and test the percent cell recovery after thawing. Most important we will test the quality of the stem cells to perform in multilineage differentiation assays. We will use the optimal human MDSC phenotype (identified in DOD 2005-2007) to identify which processing procedures are optimized for maintaining stem cell quality and quantity.

Progress: We have identified the optimal MDSC population as presented above and we have examined several cryoprotectants agents to examine the effect of freezing on the phenotype and cell viability. Human muscle stem cells which may be used in a clinical setting for cell therapeutics will need to be harvested, processed in a GMP facility, and cryopreserved prior to use. Each of these steps will expose the cells to reagents which may damage the cells or induce phenotypic changes. In addition, each step may occur at a different temperature, and the stem cells are therefore exposed to temperature cycles which are potentially damaging.

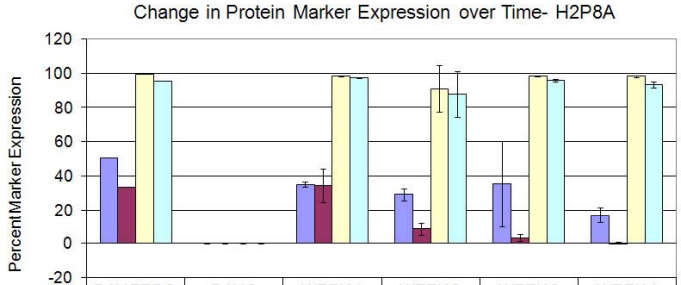
We currently collaborate with a local biotech company CookMyosite which studies human muscle-derived cells for use in urinary incontinence. In fact this company is currently conducting clinical trials transplanting these cells to the urethra of patients with urinary incontinence. In the Deasy lab, we have studied the effect of cryopreservation on these cells. In these studies, human muscle stem cells were added to a solution of fetal bovine serum (FBS) and a cryoprotectant (DMSO or glycerol) at 100,000 cells per cryopreservation vial. We examined 6 timepoints (day 0, day 2, week 1-4), done in triplicate. At the given timepoints, cells are slow frozen in the -80°C freezer, then quick

A



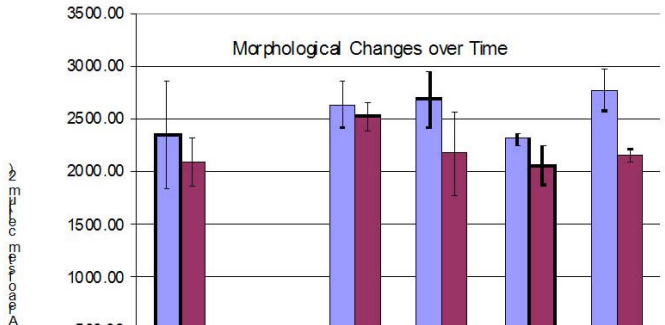
	DAY ZERO	DAY 2	WEEK 1	WEEK 2	WEEK 3	WEEK 4
H2 P8 A- 10% DMSO	100	98.41269841	84.01292335	75.13597514	78.93939394	87.24747475
H2 P8 B- 20% DMSO	100	61.02693603	61.81318681	61.80555556	65.27777778	73.21428571

B



	DAY ZERO	DAY 2	WEEK 1	WEEK 2	WEEK 3	WEEK 4
CD 56	50.57	0	34.94	29.2033333	35.1866666	16.9066666
CD 146	33.27	0	34.35	8.46666666	3.31	0.30333333
CD 73	99.36	0	98.38	90.95	98.3533333	98.16
CD 105	95.57	0	97.31	87.86	95.78	93.3266666

C



	DAY ZERO	DAY 2	WEEK 1	WEEK2	WEEK 3	WEEK 4
H2P8 A- 10% DMSO	2357.32	0.00	2640.07	2685.01	2314.12	2775.48
H2P8B-20% DMSO	2090.50	0.00	2530.19	2174.56	2061.97	2155.19

Figure 4. We have alrea dy examined the effect of DMSO and glycerol on the viability and stability o f another cell type which is being using in human clinical trials for urinary incontinence (muscl e derived cells, MDCs, Cook Myosyte). In this collaborative study, we o bserve the b est post-thaw recovery after freezing with 10% DMSO and compared to 20% DMSO (glycerol not shown)

thawed in the room temperature waterbath. For each time point , cells are thawed from cryopreservation vials, counted for viability via Trypan Blue, put in culture for 24 hours, imaged in T-25 plates, then passaged, counted again for viability via Trypan Blue (**Fig.4a**) . Remaining cells are flowed through the flow cytometer to determine percent marker expression (**Fig.4b**). Images of cells in cultured in T-25 analyzed in Image J for morphological changes (**Fig.4c).**

Under the conditions studied here, we observe a moderate about of cell death which could be improved by using higher concentrations of DMSO. Slow freezing stem cells kills around 50% cells from day 0 to week 4. We observe that10% DMSO is a better concentration than 20%- provides increased cell viability. Thawed muscle cells lost their expression of protein markers CD56 and CD146. H2P9A exhibited a 67% decrease in CD56; 99% decrease in CD146. H2P9B exhibited a 68% decrease in CD56; 97% decrease in CD146. H2P8A cell area increased by 18%; H2P8B cell area increased by 3%. There also was a significant difference in the number of cells remaining at week 4 compared to day 0, and there was significant difference in the percent marker expression of CD56 and CD146 comparing day 0 to week 4 ($p < 0.05$). There is no significant difference in area between day 0 data and week 4 for both samples.

In this Phase I project, we will focus on the effect of cryopreservation on human umbilical cord derived cells. Currently, dimethyl sulfoxide, DMSO, is the cryopreservation reagent utilized in the preservation of these cells. The cells are normally frozen in a solution of 10% DMSO, and stored at -80 C or -196 C, for days, weeks, or months. For clinical applications, human MDSCs have been shipped in a frozen state from the processing facility to the clinic(*56, 116*). It is key that these cells retain their stem cell phenotype after thawing.

Recommended changes: In the original protocol, we proposed to examine the effect of temperature fluctuations of cell phenotype. We propose to continue with these aims. We also have no recommended changes to the study of cryopreservation of the human muscle-derived cells.

Publications which have resulted from this work:

1. Chirieleison SM, Schugar RC, Deasy BM. 2008. Current Progress in Cell-mediated Gene Therapy for Muscular Dystrophies. *Research Advances in Gene Therapy*. Global Research Network. Editor: RM Mohan.
2. Schmidt, BT, Feduska, JM, Witt, AM, and BM Deasy. 2008 Robotic cell culture system for stem cell assays. *Industrial Robot*. *35 (2):116-124.*

Abstracts that have resulted from this work :

1. Chirieleison SM , Scelfo CC, Askew Y, Deasy BM. 2008. Development of Morphological Measurement Scheme Using Open Source Software for Live Cell Imaging Approaches to Stem Cell Biology. University of Pittsburgh Science 2008; Pittsburgh, PA, USA. POSTER

2. Chirieleison SM , Scelfo CC, Deasy BM. 2008. Quantitative Analysis of Therapeutic Muscle Cell Populations through Live Automated Cell Imaging. American Society for Cell Biology Annual Meeting; San Francisco, CA, USA. POSTER

3. Chirieleison SM , Scelfo CC, Askew Y, Deasy BM. 2008. Development of Morphological Measurement Scheme Using Open Source Software for Live Cell Imaging Approaches to Stem Cell Biology. Pittsburgh Orthopaedic Journal; Pittsburgh, PA, USA.

4. Scelfo CC, Chirieleison SM , Deasy BM. 2008. Integration and Adaptation of Live Cell Image Analysis. Pittsburgh Orthopaedic Journal; Pittsburgh, PA, USA.

5. Chirieleison SM , JM Feduska, RC Schugar, SL Sanford, J Huard, BM Deasy. 2008. Identifying Populations of Human Muscle Derived Stem Cells to Participate in Skeletal Muscle Regeneration Based on Phenotypic Differences. Pittsburgh Orthopaedic Journal; Pittsburgh, PA, USA.

6. Chirieleison SM , Feduska JM, Schugar RC, Witt AM, Deasy BM. 2008. In Vitro Aging of Human Muscle Stem Cells Due to Culture Expansion. International Society for Stem Cell Research; Philadelphia, PA, USA. POSTER

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9. Chirieleison SM , Feduska JM, Schugar RC, Sanford SL, Huard J, Deasy BM. 2008. Identifying Populations of Human Muscle Derived Stem Cells to Participate in Skeletal Muscle Regeneration Based on Phenotypic Differences. Orthopaedic Research Society; San Francisco, CA, USA. POSTER

Project # 5
Final Report
Inhibiting cell death and promoting muscle growth for congenital muscular dystrophy
(Xiao Xiao)

Introduction

Congenital muscular dystrophy (CMD) is a group of severe forms of muscular dystrophy leading to early death in human patients(1-3, 7). The majority of cases are caused by genetic mutations in the major laminin, laminin containing the α2 chain (formerly named merosin), in the muscle basement membrane(4, 6, 8, 11). The early morbidity/fatality and the lack of effective treatment require urgent search for novel therapeutics. Previously, we utilized mini-agrin, which has been proven to have a therapeutic effect in transgenic MCMD mice, to treat MCMD mice by AAV vector(10). Our preliminary studies showed that over-expression of mini-agrin protein greatly improved general health and muscle morphology in MCMD mice. However, the treated disease mice still developed gradual paralysis and displayed shorter life span than wild type mice. To further improve the current gene therapy paradigm, we have developed complementary gene therapy strategy for laminin alpha2-deficient CMD.

Body

As mentioned earlier, we utilized laminin alpha2 deficient congenital muscular dystrophy (MDC1A), a severe form of muscular dystrophy animal model, as our muscle injure model in this proposal. We evaluated the therapeutic effects of delivering different molecules, such as insulin-like growth factor 1 (IGF-1), anti-apoptotic gene BCL-XL, as well as myostatin propeptide, on muscle and nerve pathogenesis of MDC1A. While we did not observe the significant therapeutic effects by single delivery of those factors on MDC1A mice, we made very interesting discovery during those experiments and wrapped up two manuscripts. Since the first manuscript has been published {Qiao, 2008 #980}, we will mainly concentrate on the second manuscript in this report.

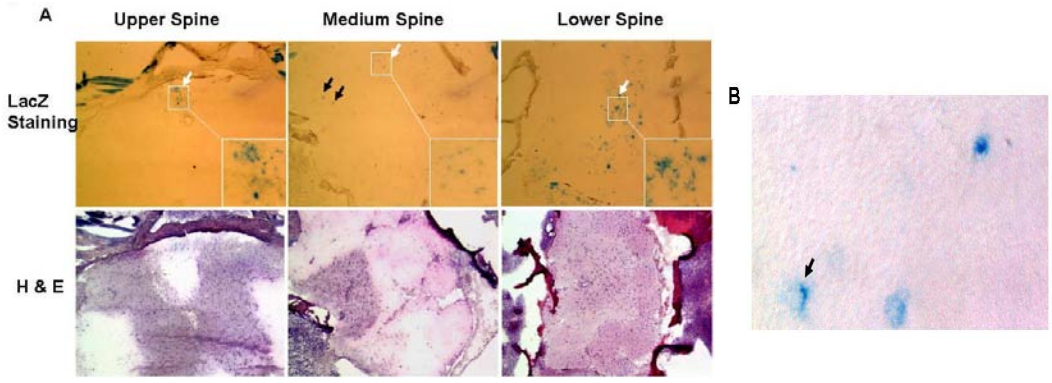

Figure 1. LacZ staining of spinal cord from AAV8-LacZ injected mice. A. LacZ positive cells in white matter. The vector was delivered on 3-day-old neonatal mice, and the mice were sacrificed at 2-month-old age. Notably, most of the lacZ positive spots were in white matter (white arrow), and very few LacZ positive cells were in grey matter (black arrow). Insets were indicated enlarged area. The consecutive sections of H&E staining were used to display the structure of spinal cord. It was also apparent that there were more LacZ positive cells in lower spine, followed by medium spine, and then upper spine. B. Few LacZ positive cells in grey matter. Button-like structure (black arrow) in grey matter indicated small neuron and axon communication.

AAV vector transduced white matter of spinal cord

Previously, we have revealed that delivering AAV serotype 8 vectors to neonatal mice can efficiently transduce whole-body skeletal muscle and cardiac muscle. In the present study, we utilized the same AAV serotype and similar delivery route to investigate the ability of AAV vector to infect peripheral nervous system and spinal cord. For the first set of study, we utilized AAV8-CMV-LacZ vector. Two months after vector injection, the mice were sacrificed. The whole spine including bone and adjacent muscle were cut into three pieces and quick-frozen away. After cryo-section, the slides were subjected to LacZ staining, and H& E staining were performed on the consecutive sections to display the histology. As shown in figure 1A, most LacZ positive cells were located in white matter in the

spinal cord of AAV8-LacZ delivered mice (white arrow and inset area). Occasionally, we could see few LacZ positive cells in grey matter (black arrow and figure 1B). Notably, there were more LacZ positive cells in the lower spine and less LacZ positive cels in the higher spine potion. Large magnification of few LacZ positive cells in grey matter of ventral horns showed button-like structures in close proximity and overlapping neuronal cells (Fig 1B). AAV8 vector effectively transduced dorsal root ganglion and peripheral nerves

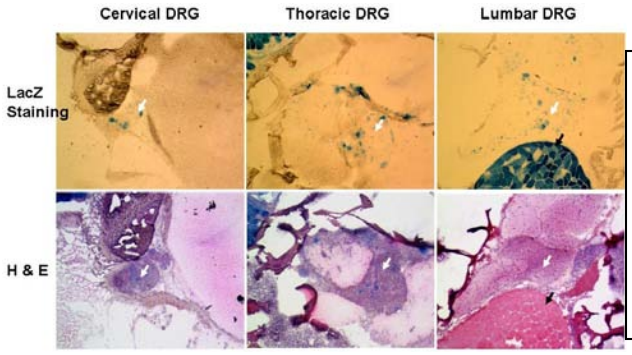

Figure 2. LacZ staining indicated AAV8-LacZ vector efficiently transduced dorsal root ganglion (DRG). White arrow indicated DRG, and the consecutive section of H&E staining were utilized to display the DRG structure. Black arrow pointed to adjacent LacZ positive muscle cells

In addition to the white matter, we noticed AAV8 vector could efficiently infect dorsal root ganglion (DRG) cells which were positioned outside of the spinal cord (Fig2 and Fig 3, white arrow). Across the whole spine, all the DRG neuronal cells were effectively transduced by AAV vector (Fig.2).

In the second set of our study, we wished to use a different report gene, such as GFP gene, to further prove our finding. Similar to LacZ vector, AAV8-D(+)-CMV-GFP vector was delivered into neonatal mice. Two months after vector injection, the mice were sacrificed. To reduce the background staining of spinal cord, we did perfusion to remove circulating blood and fixed with 4% paraformaldehyde (under anesthesia) when the mice were sacrificed. Figure 3 displayed middle portion of the spine, most of the GFP positive cells were located in while matter of spinal cord and DRG (white arrow).

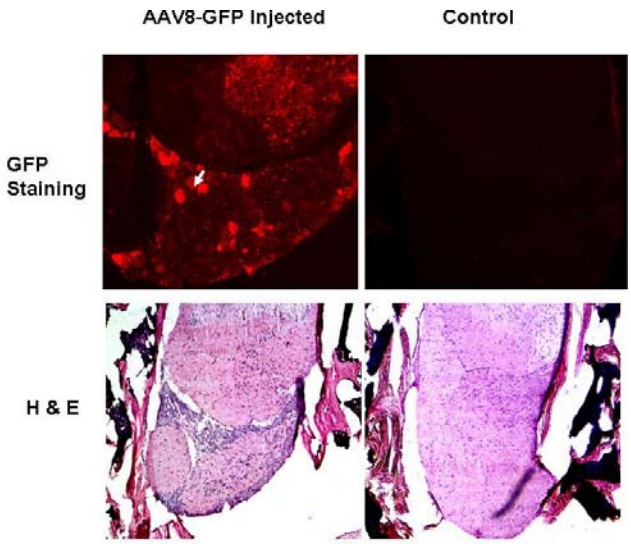

Figure 3. Immunofluorescent staining of GFP in spinal cord and dorsal root ganglion from AAV8-GFP delivered mice.

Except for white matter of spinal cord and DRG, we also observed LacZ and GFP positive cells in peripheral nerve fibers (Fig 4).

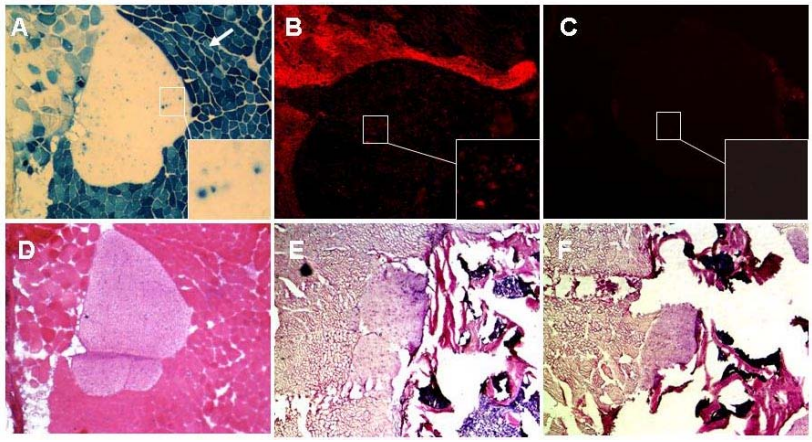

Figure 4. AAV vector efficiently transduced peripheral nerve roots. A was from AAV8-LacZ injected mice, and B was from AAV8-GFP injected mice, C was from mock injected animal but stained with GFP. D, E, F were consecutive section of H& E staining. Insects were indicated enlarged area of peripheral nerves. The LacZ positive cells surrounding peripheral nerves in picture A (white arrow) were muscle cells.

Key Research Accomplishments:

1. We have delivered myostatin propeptide gene into laminin alpha2-deficient CMD mice and the therapeutic effect was evaluated. Unlike mdx mice for which myostatin propeptide delivery can ameliorate its muscle pathology, the delivery of propeptide had a severe side effect on laminin alpha2-deficient CMD mice. The injected mice died even early than untreated controls. This phenomenon is consistent with previous discovery(5).
2. We have delivered AAV-IGF1 vector into laminin alpha2-deficient CMD mice and did not observe significant therapeutic effect.
3. We have delivered AAV-BCL-XL into laminin alpha2-deficient CMD mice and did not observe significant therapeutic effect.

Reportable Outcomes:

Our manuscript entitled “Myostatin propeptide gene delivery by AAV8 vectors enhances muscle growth and ameliorates dystrophic phenotypes in *mdx* mice” has been published in Human Gene Therapy 2008 Mar; 19(3): 241-54(9).

Our second manuscript titled “Efficient gene transfer to dorsal root ganglion and peripheral nerves via systemic delivery of AAV vectors” is in preparation and will be submitted soon.

Conclusions:

Single AAV-IGF1, AAV-Bcl-XL, or AAV-MPRO gene delivery can not offer therapeutic benefit for laminin alpha 2 deficient dy^w/dy^w mice.

AAV can efficiently transduce peripheral nervous system and dorsal root ganglion cells.

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Project # 6
Final Report
Treatment for Muscle Wasting
(Paula Clemens)

Introduction:
The ability to promote muscle regeneration in the setting of focal or generalized muscle loss could confer significant clinical benefit in the setting of focal neuropathic or other processes that cause muscle atrophy or chronic illnesses that cause cachexia. We hypothesize that gene transfer strategies can promote muscle regeneration toward a goal of improving muscle bulk and strength in the setting of injuries or diseases that cause muscle atrophy.

Extensive evidence has shown that higher levels of some pro-inflammatory cytokines contribute to the development of cachexia. For example, serum tumor necrosis factor α (TNFα) and IL-1α levels are markedly increased in patients with rheumatoid arthritis and cancer. Other cytokines, such as IL-6, IL-1β and proteolysis-inducing factor (PIF) have been reported to contribute to the development of muscle wasting.

Elegant studies show that TNFα binds to its receptor on skeletal muscle resulting in the activation of nuclear factor κB (NF-κB) mediated by phosphorylation and degradation of the NF-κB inhibitory protein, IκBα. Downstream effects of pathological NF-κB activation in skeletal muscle include the inhibition of new muscle formation and the degeneration of existing muscle. *In vitro* studies support the potential that the IκBα superrepressor (IκBSR), an IκBα genetically engineered to prevent its phosphorylation, can prevent the activation of NF-κB in skeletal muscle and could ameliorate or prevent muscle wasting. Our preliminary studies demonstrate the novel determination of inhibition of activation of NF-κB by cellular FLIP (cFLIP).

Our hypothesis is that the inhibition of the downstream pathways of TNFα causing muscle atrophy and failure of muscle regeneration should be effected intracellularly in muscle fibers. A gene therapy strategy is ideal for the purpose of achieving an ongoing effect that is cell-type restricted. We anticipate that inhibition of NF-κB activation restricted to muscle fibers will provide a therapeutic effect on muscle while avoiding potential toxic effects of inhibiting NF-κB activation in other tissues.

Body:
To characterize an *in vitro* model of cancer-induced muscle wasting in primary muscle cells and in stable muscle cell lines expressing IκBSR or cFLIP.
We developed a new *in vitro* cell culture assay to study the effects of cancer cell cytokines on muscle cell differentiation and use this assay to test novel gene transfer approaches for the treatment of cancer cachexia. Exposure to conditioned media from selected human cancer cell lines resulted in failure of muscle cell differentiation. A known intracellular mechanism of NF-κB activation as a cause of cancer cachexia was recapitulated in this *in vitro* system. We observed a direct correlation between NF-κB activation and inhibition of myogenic differentiation in the *in vitro* assay. Exposure to inflammatory cytokines and to conditioned media from human cancer cells each resulted in NF-κB activation within primary muscle cells. Failure of myogenic differentiation and the associated activation of NF-κB were prevented by stable expression of either IKBSR or cFLIP, but not by Bcl-xL. These findings were published. As described below, we used the PC-3 cell line to generate a new *in vivo* cancer cachexia model in mice.

Taken together, this study provides an *in vitro* assay that demonstrates secretion of cachexia-inducing factors by certain cancer cell lines that result in the inhibition of myogenic differentiation by activation of NF-κB. Over-expression of cFLIP in muscle cells inhibits both NF-κB-mediated and apoptotic pathways, thereby preventing

tumor media-induced inhibition of myogenic differentiation and cytotoxicity. These findings point toward the potential to design novel molecular therapeutics for the treatment of cancer-induced muscle wasting.

To clone, rescue, and purify adeno-associated viral (AAV) vectors carrying IκBSR or cFLIP.
We are generating vectors with either ubiquitous or muscle-specific promoter control of expression. AAV serotype 8 vectors provide high levels of muscle transduction and the ability to transduce muscle by systemic delivery. Where possible, we are using self-complementary vectors that result in the highest possible levels of transgene expression.
At our last report, we had rescued the following vectors as high-titer, purified AAV8 vectors: Conventional single strand AAV8 vectors were rescued with the following expression cassettes: CMV-GFP and CMV-cFLIP. Self-complimentary (double strand) AAV8 vectors were rescued with the following expression cassettes: CMV-IκBSR and MCK-GFP. The double strand MCK-GFP vector has been tested by direct intramuscular injection showing high levels of transgene expression.

Since the last report, we rescued the MCK-IκBSR double strand vector as AAV8. The MCK-cFLIP double strand vector plasmid was difficult to clone, but we finally achieved it. We are currently rescuing that plasmid as AAV8. We observed widespread skeletal muscle expression from the MCK-GFP AAV8 vector after systemic delivery. We also observed skeletal muscle expression after intramuscular and systemic delivery of the MCK-IκBSR AAV8 vector.

Characterization of a new PC-3 cell cancer cachexia model in mice.
We have refined our model of muscle cachexia in mice in order to test molecular therapeutics *in vivo*. We generated a novel model of cancer cachexia induced by PC-3 cells and compared it to an established model induced by MCA-26 cells. We reported initial findings on the *in vivo* PC-3 cell cancer cachexia model in our last report. We have now completed those studies and prepared a manuscript for submission. We showed that tibialis anterior and quadriceps muscles had increased activation of NF-κB in the PC-3 cell model of cancer cachexia, as predicted from our *in vitro* studies exposing muscle cells in culture to supernatant from PC-3 cells. Interestingly, the gastrocnemius, which did not show an increase in activated NF-κB, instead showed increased levels of phosphorylated eukaryotic initiation factor 2 alpha (p-eIF2-α) indicating decreased protein synthesis contributing to the cachexia phenotype. This raises the interesting possibility that increased protein degradation or decreased protein synthesis may contribute to cachexia to different degrees in different muscle groups.

To apply AAV8 vector strategies to ameliorate cancer cachexia.
Five-week old CD2F1 mice received intramuscular administration of either AAV8-MCK-dsIκBSR or AAV8-CMV-dsIκBSR or AAV8-CMV-ss cFLIP vector 1 week prior to MCA-26 tumor inoculation. Body weight and tumor growth were monitored every other day. All mice developed tumors and cachexia beginning at about 12 days after tumor inoculation (Figure 1). Three weeks tumor after innoculation the mice were sacrificed and hind-limb muscles were dissected, weighed, and snap-frozen for analysis. The tibialis anterior muscle injected with AAV8-MCK-dsIκBSR showed a significant increase in weight compared to tumor-bearing control (Figure 2). Tibialis anterior muscle injected with each of the 3 therapeutic AAV8 vectors showed a significant increase in muscle fiber diameters compared to tumor-bearing control (Figure 3).

Our results suggest that vectors expressing proteins with the intended effect of decreasing NF-κB activation can ameliorate cancer cachexia *in vivo*. Future studies are needed to test vector-mediated gene delivery of IκBSR and cFLIP by systemic delivery in cancer cachexia models.

Figure 1A

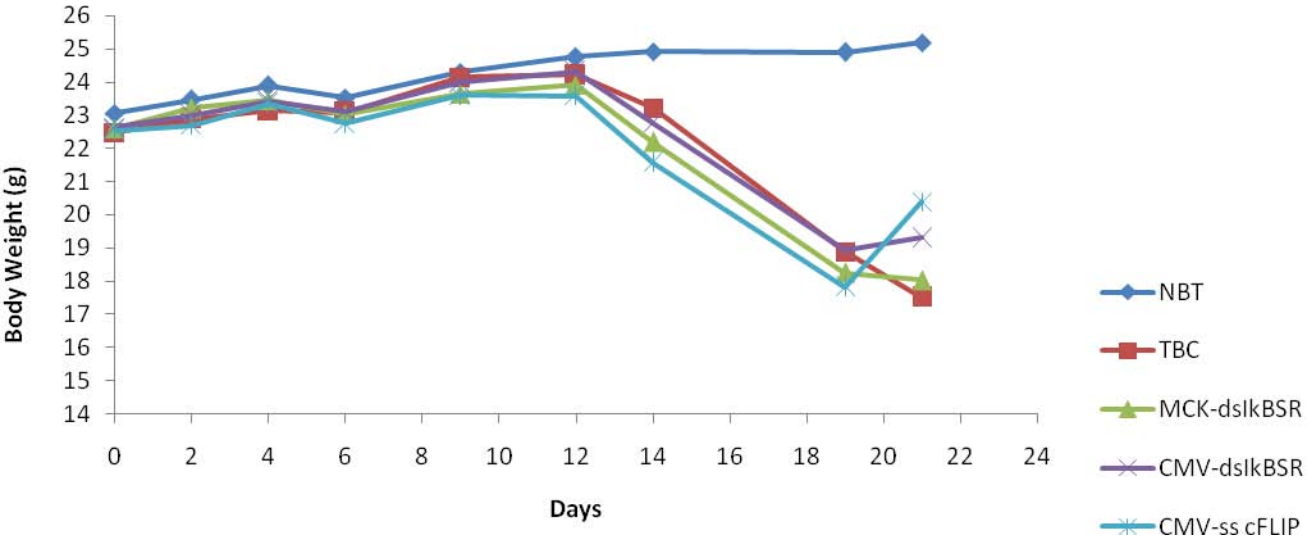

Figure 1B

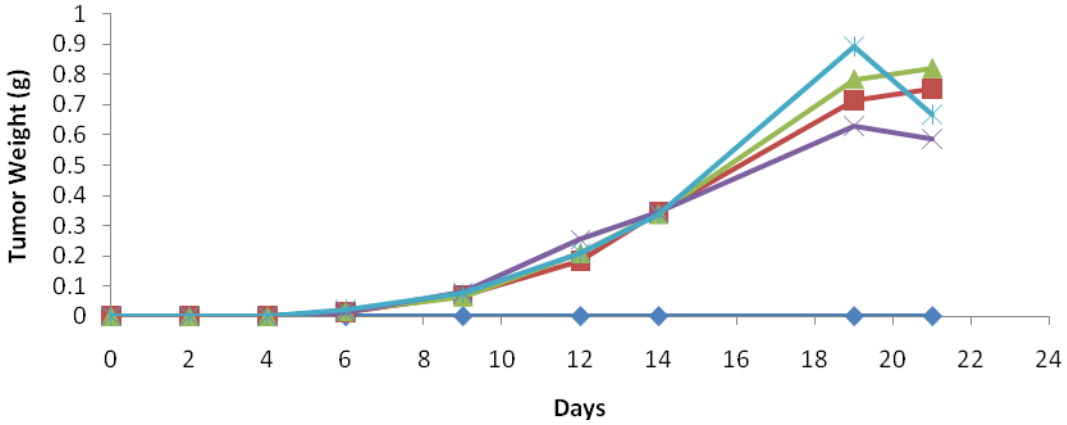

Figure 1. Effect of intramuscular administration of AAV8 carrying MCK-I BSR or CMV-I BSR or CMV-cFLIP on body and tumor weight of MCA-26 tumor-bearing in comparison with non-tumor-bearing control (NTB)

CD2F1 mice were intramusculary injected with AAV8 vector carrying MCK-I BSR or CMV-I BSR or CMV-cFLIP on tibialis anterior and quadriceps muscle ($3.4x10^{10}$ v.p./muscle) and one week after the mice received MCA-26 tumor inoculation (10^6 cells/mouse) (day 0). (A) Body weight change of mice bearing MCA-26 tumor with or without AAV8 treatment as compared to NTB. (B) Tumor growth rate in MCA-26 tumor-bearing mice with or without AAV8 treatment. The number of mice in each group n=5.

Figure 2

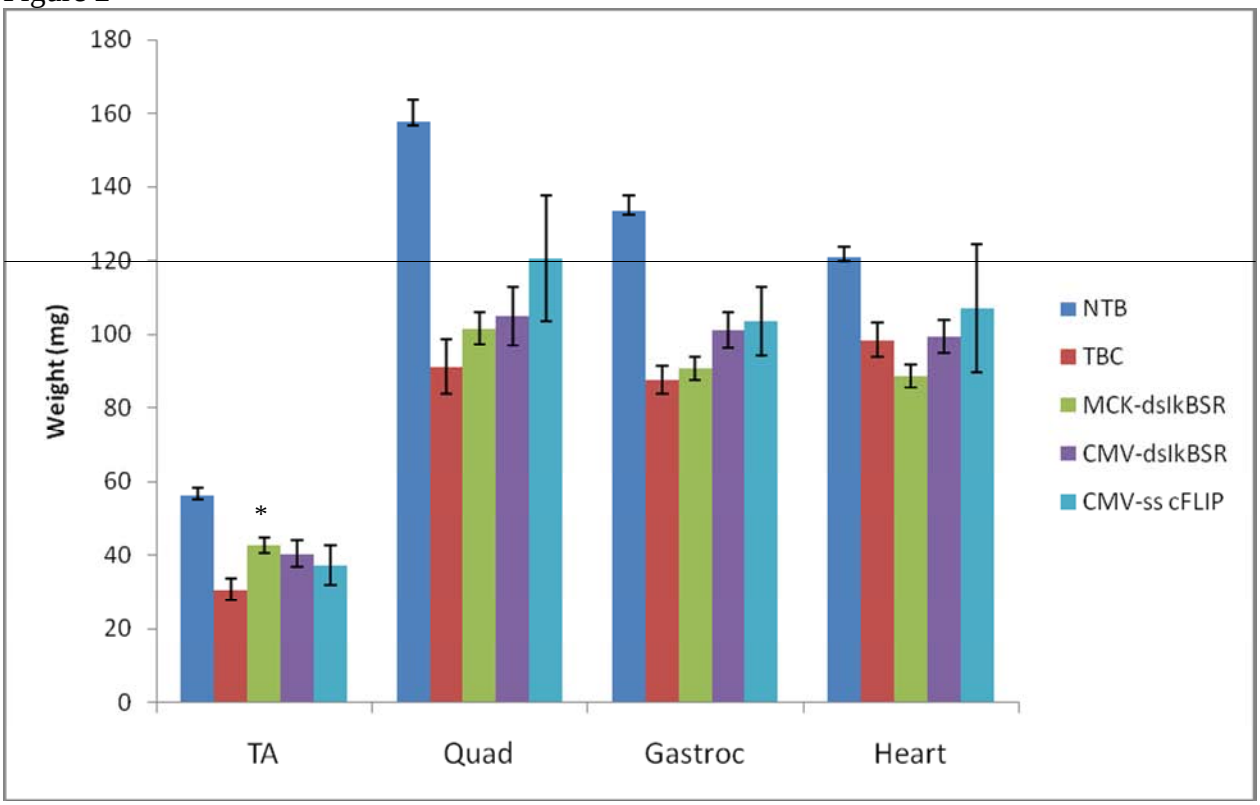

Figure 2. Treatment with AAV8 carrying MCK-I BSR or CMV-I BSR or CMV-cFLIP results in improved muscle weight in mice bearing MCA-26 tumor

Mice were sacrificed and hind-limb muscles (TA = tibialis anterior, Quad = quadriceps, Gastroc = gastrocnemius) and heart were collected. The average weight of each individual muscle of NTB and MCA-26 tumor-bearing mice with or without AAV8 treatment are shown. Differences from tumor-bearing without AAV8 treatment (TBC = tumor-bearing control) are shown * = $p < 0.05$.

Figure 3A

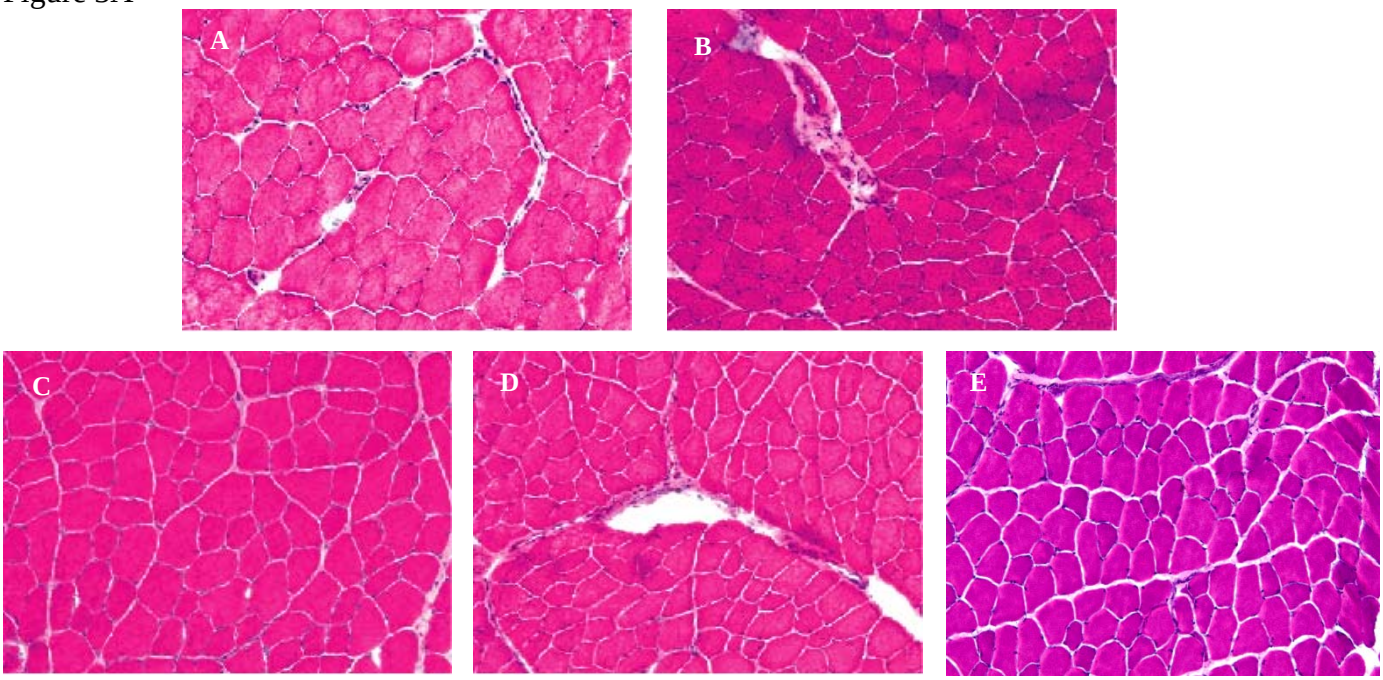

Figure 3B

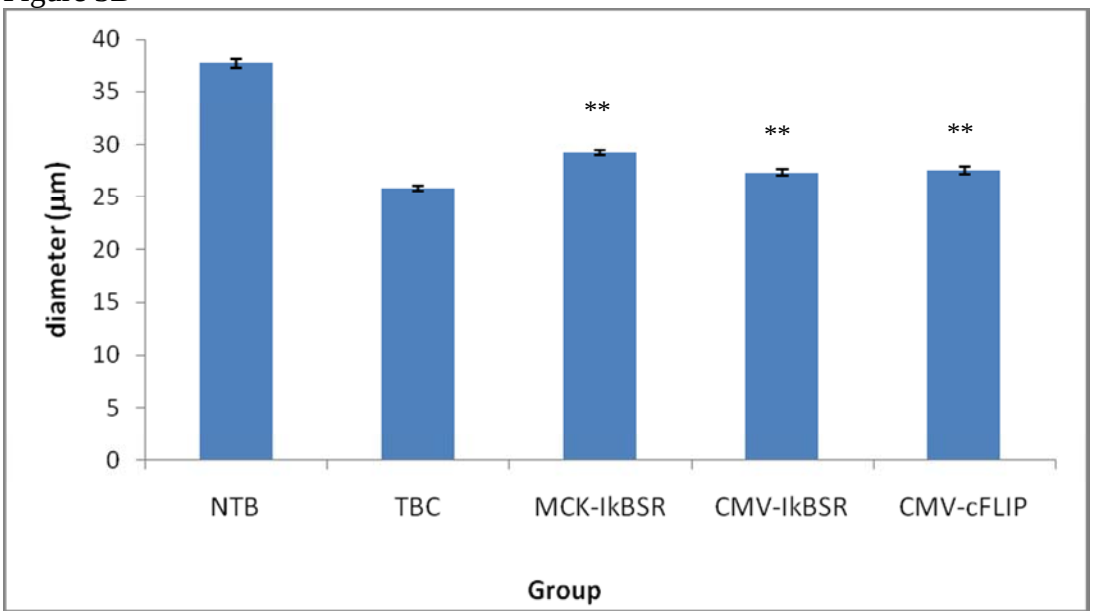

Figure 3. Treatment with AAV8 carrying MCK-IκBSR or CMV-IκBSR or CMV-cFLIP results in increased muscle fiber diameters in mice bearing MCA-26 tumor
Representative H&E staining of TA muscle section is shown in (A); (a) non-tumor-bearing control, (b) MCA-26 tumor-bearing control, (c) AAV8-MCK-IkBSR injected MCA-26 cachexia, (d) AAV8-CMV-IkBSR injected MCA-26 cachexia, (e) AAV8-CMV-cFLIP injected MCA-26 cachexia. The average muscle fiber diameter of each group is shown in (B). Differences from TBC (tumor-bearing control) are shown ** = $p < 0.01$.

Key Research Accomplishments:

- Development of an *in vitro* assay of muscle wasting induced by cancer cytokines
- Demonstration of importance of NF-κB activation as a molecular mechanism for cancer-induced muscle wasting in the *in vitro* assay
- Use of the *in vitro* assay to identify cFLIP as a novel potential therapeutic agent for the treatment of cancer-induced muscle wasting
- Generation of single strand and double strand AAV8 vectors
    - Single strand AAV8 vectors
        - CMV-GFP
        - CMV-c-FLIP
    - Double strand AAV8 vectors
        - MCK-GFP
        - CMV-IκBSR
        - MCK-IκBSR
    - Cloned as plasmid
        - AAV-MCK-D(+)-c-FLIP
- Refinement of 2 cancer cachexia models in mice
- Testing of AAV8 vectors carrying IκBSR and cFLIP for amelioration of cancer cachexia in an *in vivo* murine model

Reportable Outcomes:

Jiang Z, Clemens PR. Cellular caspase-8-like inhibitory protein (cFLIP) prevents inhibition of muscle cell differentiation induced by cancer cells. FASEB J 2006 Dec;20:E1979-E1989.

Sae-Chew P. Clemens PR. A Novel Muscle-Wasting Mouse Model Induced By Human Prostate Cancer Cells: Comparison with a Well Established Model Induced By Murine Colon Adenocarcinoma Cells. Prepared for submission (attached).

Conclusions:
Our studies to date identify an *in vitro* assay that allows us to test molecular therapies that have the potential to treat muscle wasting induced by cancer. We anticipate that these results can be generalized to the treatment of other genetic and acquired causes of muscle wasting. We produced multiple AAV8 vectors with expression cassettes designed to inhibit activation of NF-κB and ameliorate cancer cachexia. We applied several newly developed AAV8 vectors for treatment of cancer cachexia in a murine model.

Vector Core
Progress Report
(Bing Wang)

Vector Core of the DOD project (W81XWH-06-1-0406)
Sub-title: The structure, production and purification of adeno-associated viral, adenoviral and retroviral vectors"

Introduction:

The Molecular Therapy Laboratory (MTL) in the Department of Orthopaedic Surgery is focusing on viral based gene transfer and stem cell technologies for musculoskeletal diseases, mainly by using recombinant adeno-associated viral (rAAV), retro-viral and lenti-viral vectors which represent the most promising approaches to aid in the repair and regeneration of muscle, bone, ligament, tendon, and joint capsules. In the meanwhile, MTL also serves as a Vector Core of a DOD project (W81XWH-06-1-0406, PI: Dr. Johnny Huard) for the design, construction and production of a lots of viral vectors which are used to gene transfer and genetic engineering of stem cells in the whole project.

The Vector Core is located in the Biomedical Science Tower, room E1603. Total dedicated Vector Core space is approximately 500 square feet for molecular biology and viral vector production. Inventory of major equipment includes one tissue culture hood and one temperature-controlled CO2 incubator for cell culture and viral vectors production (**Figure 1 A**). In addition, Vector Core is also equipped with one Beckman TM L-100 XP Ultracentrifuge, one Beckman Avanti TM J-20 XP high-speed centrifuge for the purification of plasmid DNA and viral vectors (**Figure 1B**). Moreover, one Beckman refrigerated table-top centrifuge, two microfuges, one refrigerator, one BioRad PCR machine, one UV spectrophotometer, and one BioRad gel documentation camera (**Figure 1C**), and two BioRad electric gel transfer apparatuses will be used for molecular analysis and vectors construction. Also, one Nikon inverted fluorescent microscope and -30ºC and -80ºC freezers, and one liquid nitrogen tank are established for evaluating the expression of report genes, storages of plasmid DNA, viruses and cell clones.

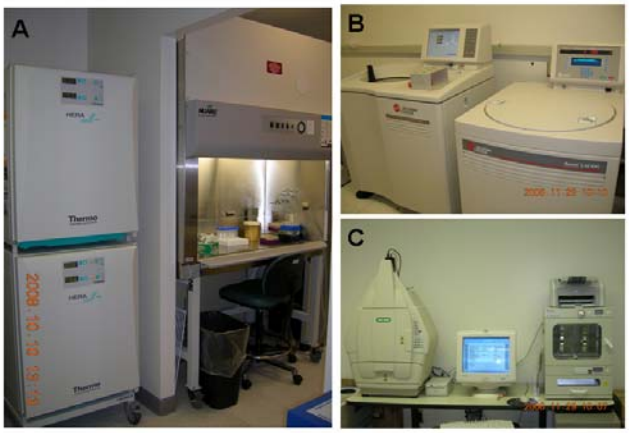

Figure 1. Major Equipment

Body

Development and production of AAV vectors

The Vector Core is actively engaged in multiple applications of rAAV vectors for gene therapy, including different serotypes of AAV vectors, both single and self-complementary AAV vectors, inducible vector system. Moreover, we developed the different promoters in AAV vectors and gene-silencing AAV vectors. As a vector core, we collaborated with many laboratories internal and external the University of Pittsburgh. Our goal is to develop very useful viral vectors for directly gene transfer and to aid in the characterization of the cells, track the cells and genetically modify the cells to express beneficial proteins to enhance their transplantation efficiency. Such as Decorin, mini-dystrophin, and myostatin propeptide genes etc. were constructed in AAV vectors. As described in following, we produced lots of AAV vector for the aims in this project:

1. AAV2-D(+)-CMV-eGFP
2. AAV6-D(+)-CMV-eGFP
3. AAV8-D(+)-CMV-eGFP
4. AAV9-D(+)-CMV-eGFP

5. AAV8-D(+)-CMV-eGFP
6. AAV8-D(+)-tMCK-eGFP
7. AAV2-D(+)-CB-eGFP
8. AAV2-1060-hrGFP
9. AAV2-1070-hrGFP
10. AAV2-1060-Gluciferase
11. AAV2-1070-GLuciferase
12. AAV2-CMV-sflt1
13. AAV2-D(+)-BMP2-spA
14. AAV2-D(+)-BMP2-SV40
15. AAV2-D(+)-CMV-TGFβ1
16. AAV2-CMV-minidystrophin (3858)
17. AAV2-dMCK-minidystrophin (3858)
18. AAV6-D(+)-CMV-BMP4
19. AAV2-D(+)-CMV-VEGF
20. AAV1-CMV-Decorin

Development and production of lenti-viral vectors

In the meanwhile, we also updated the BSL-2 to BSL-2+ for development of lenti-viral vectors using in the aims of this project, including green (GFP) and red (RFPN) reporter genes and silencing lenti-viral vectors targeting to human and mouse VEGF genes.

1. lenti-CMV-GFP
2. lenti-CMV-RFPN
3. lenti-CMV-Luciferase
4. lenti-minidytrophin (3849)-GFP
5. lenti-shRNA-mouse VEGF-GFP
6. lenti-shRNA-human VEGF-GFP

Efficiency of viral vectors

To test the efficiencies of AAV and lenti-viral vectors, we made different viral vectors and the sequential dosages of reporter viral vectors to test which viral vectors could achieve the higher transduction efficiency and lower dose vector loading, such as green fluorescent protein (GFP) and red fluorescent protein in nucleolus (RFP-N), as shown in **Figure 3**. Also, we used same strategy to optimize the serotype of AAV vector that appears a variety of AAV transduction in the different cells and tissues. The advantage of the GFP or RFP reporter is that it can be directly visualized on the whole limb as well as on the cryo-sections. Its fluorescent intensity could also be quantitated by the Nikon microscope imaging software we have been using in the lab. Usually, at 3 weeks following implantation, at which time the GFP gene expression is expected to reach a considerable level for detection and quantitation, the animals would be sacrificed and skeletal muscles, hearts and liver will be collected for photographs for GFP or RFP expression and for microscopic photographs and quantitations from the intensity of the fluorescence after cryo-thin-section.

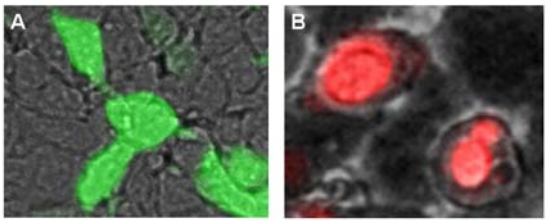

Figure 3. Transduction of AAV- and lenti-viral vectors in 293 cultured cells 36 hours post-infection. (A) shows AAV-GFP, and (B) represents Lenti-RFP-N

To achieve *ex vivo* gene transfer into *mdx* skeletal muscle using lenti-minidystrophin gene, we constructed a fusion protein containing minidystrophin and GFP reporter gene. GFP reporter can monitor the proliferation and differentiation of muscle derived stem cells *in vivo*, and it can also represent the expression of mini-dystrophin gene directly under microscopy, without toxicity and immune response. In preprimary study, we created this fusion protein construct in a pEGFP-N1 plasmid, and then cloned it into lenti-viral vector under the control of the CMV promoter. As shown in **Figure 4**, the fusion protein was expressed in both C2C12 and 293 cells after transfection.

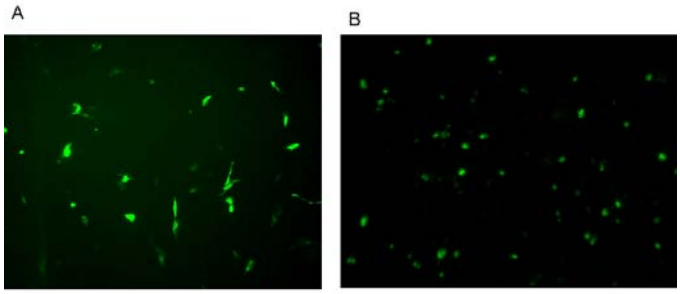

Figure 4. The expression of mini-dystrophin-GFP fusion protein after transfection. (A) 24 hours post-transfection of Mini-dystrophin-GFP fusion in C2C12 by Biotex. (B) 4 days post-transfection of Lenti-Mini-dystrophin-GFP fusion in 293 cells,

Achievements

Invited review book "Regenerative therapy for the musculoskeletal system using recombinant adeno-associated viral vectors"

1. **Bing Wang** and Johnny Huard, 2008, the Chapter "Gene therapy for the treatment of muscle disorders"
2. **Bing Wang** and Freddie H. Fu, 2008, the Chapter "rAAV vectors for soft tissue reconstruction"

Publication and abstracts in 2008 and 2009

1. Chunping Qiao et al. *Human Gen Therapy, 2008, 19 (3), 241-253.*
2. Bing Wang et al. *Gene Therapy, 2008, 15 (15), 1099-1106.*
3. Bing Wang et al. *Gene Therapy, 2008, 15 (22), 1489-1499, correspondence author.*
4. Juan Li etal. *Hum Gene Ther. 2008, 19:958-964.*
5. Bing Wang et al. *Journal of Orthopaedic Research, 2009,27:421-426, correspondence author.*
6. Bin Li et al. *Journal of Biomechanics, 2008, 41:3349-3353.*
7. Michael Y. Mi et al. *Molecular Therapy, 2008, vol.16, suppl 1, S103, correspondence author.*
8. Peiqi Hu et al. *Molecular Therapy, 2008, vol.16, suppl 1, S201*
9. Bhanu M et al. *Molecular Therapy, 2008, vol.16, suppl 1, S293*
10. Joseph Kornegay et al. *Molecular Therapy, 2008, vol.16, suppl 1, S374*
11. Ying Tang; Bin Li, James H-C Wang, Johnny Huard, Melessa Salay, Bing Wang. *In vitro* AAV-mediated gene transfer in human fibroblasts. *54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008*
12. Bin Li, Micheal Lin, Ying Tang, Bing Wang, James H-C Wang. Micropatterned C2C12 cells exhibit enhanced differentiation into myotubes – a novel study using cell traction force microscopy. *54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008*
13. Guangheng Li, Bo Zheng, Andres J. Quintero, Arvydas Usas, Bing Wang, Laura B. Meszaros, Karin A. Corsi, Johnny Huard. Therapeutic Utility of Heterotopic Ossification Induced by AAV-BMP4 in Skeletal Muscle. *54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008*
14. Mi, Y.M; Tang, Y; Li, G-H; Salay, M.N; Niyibizi, C; Huard, J; Wang, B. AAV Based *Ex Vivo* Gene Therapy in Rabbit Adipose Stem/Progenitor Cells for Osteogenesis. *55th Annual Meeting of Orthopaedic Research Society, February 22 – 25, 2009, Las Vegas, Nevada.*
15. Tang, Y; Reay, D.P; Salay, M.N; Mi, Y.M; Clemens, P.R; Guttridge, D.C; Robbins, P.D.; Huard, J; Wang, B. Delivery of Dominant Negative IKKs by Recombinant Adeno-associated Virus: Efficient Improvement in Muscle Regeneration in *mdx* Mice. *55th Annual Meeting of Orthopaedic Research Society, February 22 – 25, 2009, Las Vegas, Nevada.*

16. Michael Y. Mi, Ying Tang, Melisa N. Salay, Guangheng Li, Johnny Huard, Freddie H. Fu, Christopher Niyibizi and Bing Wang. *AAV ex vivo Gene Therapy for Osteogenesis and Chondrogenesis. Submitted to 12th Annual meeting of ASGT (May 27-30, 2009, San Diego, California).*

17. Daibang Nie, Dong Wei, Michael Y. Mi, Ying Tang, Allan Z. Zhao, Yifan Dai, Johnny Huard, and Bing Wang. Muscle Specific Gene Transfer of the PEPCK-C Improves Physical Activity in Mice. *Submitted to 12th Annual meeting of ASGT (May 27-30, 2009, San Diego, California).*

18. Ying Tang, Daniel P. Reay, Melessa N. Salay, Michael Y. Mi, Paula R. Clemens, Denis C. Guttridge, Paul D. Robbins, Johnny Huard, Bing Wang. AAV Based Blocking of NF-kappa B Pathway Improves Muscle Regeneration in *mdx* Mice. *Submitted to 12th Annual meeting of ASGT (May 27-30, 2009, San Diego, California).*

Awards

1. Ying Tang et al. *In vitro* AAV-mediated gene transfer in human fibroblasts. Oral presentation given at the 54th Annual Meeting of Orthopaedic Research Society, March 1st – March 6th, 2008.

2. Michael Y. Mi et al. AAV Based *Ex Vivo* Gene Therapy in Rabbit Adipose Stem/Progenitor Cells for Osteogenesis" was accepted as a podium presentation at the 55th Annual Meeting of Orthopaedic Research Society, February 22 – 25, 2009, Las Vegas, Nevada.

Conclusion

The Vector Core for this project provided a series of viral vectors containing reporter and therapeutic genes proposed in aims. Also, it will develop the Tet-On retro-viral vector system to express a VEGF-LacZ fusion protein.

Bioreactor Core Progress Report March 2009
Progress Report
(Johnny Huard and Bridget Deasy)

The Bioreactor Core continues to provide an innovative approach to cell and molecular biology studies. We utilize unique technology which enables a novel bioimaging approach. Our goal is to generate functional knowledge on cell behavior based on macromolecular changes and cell-cell interactions within the population niche. Within the past year, the Core has acquired and additional imaging system and new software to facilitate cell biology assays and analysis. The core now consists of 3 microscope systems; Nikon Eclipse TE 2000 U systems with customized robotic stages. Photo-metric ES Cool Snap cameras are capable of acquiring up to 30 frames per second. For fluorescent live cell microscopy in 6 colors, we use X-Cite 120 EXFO Fluorescence Illuminator. Core users come from several different departments at the University of Pittsburgh.

We recently described the benefits of the bioreactor core technology for stem cell assays(*1*). Automated high-throughput time lapsed imaging of stem cell cultures enhances the ability of biological researchers to conduct multivariate experiments with time dependent parameters. Time-lapsed microscopic imaging of cells has been in use for more than 50 years (*2-6*), though only recently have software advances allowed for expanded experimental capabilities in which image processing routines have been developed for the quantitative analysis of viable, growing cells (*7-11*). Previously, time lapsed imaging techniques required significant time allotments and were error prone due to less than ideal circumstances in which cells were monitored and tracked *in vitro* by hand. Also, multivariate experiments were scaled versions of typical experiments and were thus limited by the effort required to maintain them. With robotic control, high-throughput time lapsed imaging has become an ideal platform for stem cell experiments with multiple variables and measurements on time dependent parameters such as cell growth and cell velocity.

Our recent report described the robotic systems for microscopic imaging, LACI, (live automated cell imager) which is programmed by the user to observe a large number of regions of interest (ROIs) that are imaged in visible and/or epifluorescent light. The automated stage and focus are controlled by custom software. Each ROI may have its own variable condition and the imaging is repeated at user-defined time intervals. The output data are stacks of images for each ROI with the time-interval between consecutive images. A small, but growing number of investigators are developing custom time-lapsed imaging systems (*12-15*) to better study cell behavior. However, the details of such instrumentation to date has been brief and limited to short descriptions in the methods section, while focusing on assay endpoints and the challenge of image analysis (*16, 17*). In our study, we presented a full description of the LACI instrument. In addition to showing the design and features of LACI, we also focused on the design considerations that relate to the study of stem cells. We presented the results in which human stem cells of different ages were examined for cell proliferation rates, velocity and cell size in order to determine how each parameter relates to cell age, as cell age has been shown to be related to a decline in stem cell function. Each of these parameters could play a role in how stem cells perform in therapeutic treatment for disease or injuries. The advantages of a robotic time-lapsed microscopic imaging system for characterizing stem cells during *in vitro* biological assays were shown.

Key Core Users and Collaborative Projects (a sample of several core collaborations)

1. Dr. Bridget Deasy, Department of Orthopedic Surgery, University of Pittsburgh Medical Center
2. Dr. Johnny Huard, Department of Orthopedic Surgery, University of Pittsburgh Medical Center
3. Dr. Yong Li, Department of Orthopedic Surgery, University of Pittsburgh Medical Center
4. Dr. Vonda Wright, Department of Orthopedic Surgery, University of Pittsburgh Medical Center
5. Drs. Gwen Sowa and James Kang, Department of Orthopedic Surgery, University of Pittsburgh Medical Center
6. Dr. Fabrisia Ambrosio Department of Rehabilitation Sciences, University of Pittsburgh Medical Center
7. Dr. Partha Roy. Dept of Bioengineering, University of Pittsburgh

8. Dr. Alan Wells, Departments of Pathology, University of Pittsburgh

2008-2009 JOURNAL and ABSTRACT PUBLICATIONS which include Bioreactor Core Technology

JOURNAL PUBLICATIONS

1. RC Schugar, KE Wescoe, SM Chirieleison, JJ Nance, Y Askew, BT Schmidt, JM Evron, B Peault and BM Deasy. 2009. In situ Identification of CD146 Stem Cells in Primitive Human Umbilical Cord Stroma with High Isolation Yield, High Expansion and Phenotype Stability for Regenerative Therapies. *In Review.*
2. Deasy BM, Feduska JM, Li Y, Payne TR, Ambrosio F, Huard J, 2009. A Role for VEGF in dystrophic skeletal muscle tissue regeneration: VEGF increases vascularization and decreases fibrosis. *In revision.*
3. Joo Han Kim, BM Deasy, H Y Seo, RK Studer, NV Vo, HI Georgescu, GA Sowa, JD Kang. Notochordal cells in intervertebral disc have the ability to differentiate into chondrocyte-like cells *in vitro*. *Accepted*
4. F Ambrosio, RJ Ferrari, G Distefano, JM Plassmeyer, GE Carvell BM Deasy, ML Boninger, G. K Fitzgerald, J Huard. 2009. The Synergistic Effect 3 Of Treadmill Running On Stem Cell Transplantation To Heal Injured Skeletal Muscle. *In Review.*
5. Urish KL, Vella JB, Okada M, Deasy BM, Tobita K, Keller BB, Cao B Piganelli JD, and J Huard. Antioxidant Levels are a Major Determinant in the Regenerative Capacity of Muscle Stem Cells. 2008 *Epub Nov 12, 2008 PMID: 19005220 Mol Cell Biol*
6. Crisan M, Yap, S,, Casteilla L, Park TS, Chen, CW, Sun B, Zheng B, Norotte C, Corselli M, Traas J, Schugar RC, Deasy BM, Andriolo G, Buhring HJ, Lazzari L, Giacobino JP, Huard J and Peault B. Perivascular origin of mesenchymal stem cells in human organs. Cell Stem Cell 2008. 3(3):301-13
7. Schmidt BT, Feduska J, Witt A, Deasy B. Robotic cell culture system for stem cell assays. Industrial Robot 2008;35(2):116-124.

ABSTRACT PUBLICATIONS (selected list)

1. Sowa GA, Kim JH, Deasy BM, Seo HY, Studer RK, Vo NV, Georgescu HI, Kang JD. 2009. Notochordal cells in intervertebral disc have the ability to differentiate into chondrocyte-like cells *in vitro.* Orthopaedic Research Society; Las Vegas, NV, USA.
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3. Weiss C, Plassmeyer, J, Deasy BM, Chirieleison SM, Huard J, FA Ambrosio. 2009. Paracrine Influences of Normal Muscle Derived Stem Cells Enhance the Myogenic Potential of Stem Cells Isolated from Dystrophic Muscles. Orthopaedic Research Society; Las Vegas, NV.
4. Ding Z, Gau D, Deasy BM, Chirieleison SM, Roy P. 2008. Ligand interactions of profilin-1 in migration and capillary morphogenesis of vascular endothelial cells. American Society for Cell Biology Annual Meeting; San Francisco, CA, USA. *POSTER.*
5. Clarry M, Deasy BM. 2008. Differences in Male and Female Umbilical Cord Stem Cells. University of Pittsburgh CARE Program*;* Pittsburgh, PA, USA. *PODIUM*
6. Weiss C, Plassmeyer J, Witt M, Deasy BM, Chirieleison SM, Huard J, Ambrosio F. 2008. Paracrine Influences of Normal Muscle Derived Stem Cells Enhance the Myogenic Potential of Stem Cells Isolated from Dystrophic Muscles. Children's Hospital of Pittsburgh Research Symposium*;* Pittsburgh, PA, USA. *POSTER*

7. Schugar RC, Nance JJ, Schmidt BT, Deasy BM. 2008. The Role of CD146 in Proliferation and Differentiation of Umbilical Cord Derived Stem Cells. American Society for Cell Biology Annual Meeting; San Francisco, CA, USA. *POSTER*
8. Chirieleison SM, Scelfo CC, Deasy BM. 2008. Quantitative Analysis of Therapeutic Muscle Cell Populations through Live Automated Cell Imaging. American Society for Cell Biology Annual Meeting, San Francisco, CA, USA. *POSTER*
9. Scelfo CC, Chirieleison SM, Deasy BM. 2008. Integration and Adaptation of Live Cell Image Analysis. Pittsburgh Orthopaedic Journal, Pittsburgh, PA.
10. Chirieleison SM, Scelfo CC, Askew Y, Deasy BM. 2008. Development of Morphological Measurement Scheme Using Open Source Software for Live Cell Imaging Approaches to Stem Cell Biology. Pittsburgh Orthopaedic Journal, Pittsburgh, PA.
11. Schugar RC, Crisan M, Deasy BM. 2008. Proliferative differences between CD146+ and CD146- mesenchymal stem cells derived from the human umbilical cord. Pittsburgh Orthopaedic Journal, Pittsburgh, PA.
12. Chirieleison SM, Feduska JM, Schugar RC, Sanford SL, Huard J, Deasy BM. 2008. Human Muscle Derived Cells Isolated by the Preplate Technique Participate in Skeletal Muscle Regeneration Based on Phenotypic Differences in Cell Adhesion Rates During Isolation. Pittsburgh Orthopaedic Journal, Pittsburgh, PA.
13. Schmidt BT, Schugar RC, Deasy BM. 2008. Development of an Image Analysis Method to Quantitate the Biological Chondrogenic Differentiation Assay. Pittsburgh Orthopaedic Journal, Pittsburgh, PA.
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Micro-CT Core
Progress Report
(Johnny Huard and Arvydas Usas)

Dr. A. Usas operates vivaCT 40 (Scanco Medical) imaging system that enables nondestructive 2-D and 3-D visualization and quantitative analysis of mineralized matrix volume, density and other structural parameters of bone tissue. We are able to perform live animal imaging while they (mice or rats) are put asleep under inhalation anesthesia. VivaCT 40 system allows us to perform multiple scans on the same animal at different time points; therefore we are able to reduce the number of animals required to complete the experiment and cut the cost for animal housing. We can also perform imaging on organs and tissue specimens (calvaria, spine, extremities, muscles, etc.) harvested after animal euthanasia and stored in fixative solution for extended period of time.
Recently we implemented live cell pellet culture imaging that allows us to scan cell pellets for matrix mineralization and return cells back to the incubator for continuous prolonged cultivation without any noticeable harm on cell proliferation and differentiation. This system also enables us to perform screening for the best cellular candidates for bone tissue engineering.
We continue to investigate skeletal muscle vasculature using contrast-enhanced micro-CT imaging. One method that has been successfully applied involves transcardiac perfusion and injection of Microfil® silicone rubber compound. After Microfil polymerization that takes 4-6 hours in room temperature blood vessel network in post-mortem tissues of non-surviving animals becomes radiopaque and easily detectable by micro-CT. Another contrast agent for vascular imaging is Fenestra VC, which can be administered repeatedly to animals via single dose administration into tail vein. With Fenestra VC, the delayed uptake by liver cells produces an agent with superior blood pool imaging properties that last for several hours after injection. We are planning pilot experiments to investigate whether Fenestra VC can be used to visualize peripheral vasculature in living mice.

During the report period the use of micro-CT contributed to the following research accomplishments by the members of our laboratory:

1. Microcomputed tomography analysis of matrix mineralization within three-dimensional cell pellet culture of human skeletal muscle cells. Usas A, et al. Poster at the Scanco user meeting 2008, Appenzell, Switzerland.
2. Human skeletal muscle cells undergo matrix mineralization in an osteogenic pellet culture assay. Corsi K, et al. Poster at the ASBMR meeting 2008, Montreal, Canada.
3. Human skeletal muscle-derived cells undergo matrix mineralization in an osteogenic pellet culture assay. Usas A, et al. Podium presentation at the international bone-tissue engineering congress, Bone-tec 2008, Hannover, Germany.
4. Ex vivo Noggin gene therapy inhibits bone formation in a mouse model of postoperative resynostosis. Cooper GM, Usas A, Olshanski A, Mooney MP, Losee JE, Huard J. Plast Reconstr Surg. 2009 Feb;123(2 Suppl):94S-103S.
5. Small intestine submucosa facilitates the repair of critical size calvarial defects in mice. Usas A, et al. Poster at the ORS meeting 2009, Las Vegas, NV
6. CD56 positive cell population in human skeletal muscle displays the highest osteogenic potential. Li GH, et al. Poster at the ORS meeting 2009, Las Vegas, NV.
7. Muscle-derived stem cell mediated ectopic bone formation is delayed in females due to recruitment of host cells. Meszaros LB, et al. Poster at the ORS meeting 2009, Las Vegas, NV.
8. Isolation and characterization of human ACL-derived vascular stem cells in injured ACL. Matsumoto T, et al. Poster at the ORS meeting 2009, Las Vegas, NV.
9. Characterization of human meniscus-derived vascular stem cells; comparison of periphery and inner cells. Osawa A, et al. Poster at the ORS meeting 2009, Las Vegas, NV.

10. A novel accelerated aging murine model exhibiting intervertebral disk degeneration. Vo N, et al. Short talk and poster at the ORS 2009, Las Vegas, NV.
11. Scaffold-guided bone-tissue engineering in a rabbit forearm. Abstract accepted for oral presentation at the EFORT congress 2009, Vienna, Austria.
12. Generation of a clonal stem cell population from adult mouse skeletal muscle that undergoes spontaneous microenvironment-specific transformation-refining a mouse model of tumorigenesis. Barry DA, et al. Abstract submitted to ISSCR meeting 2009, Barcelona, Spain.
13. Treatment using a therapeutic peptide inhibitor against the stress mediator NF-kB delayed age-dependent spinal degeneration in an accelerated aging mouse model. Vo N, et al. Abstract submitted to NASS meeting 2009, San Francisco, CA.
14. Myoendothelial cells: a novel stem cell population from adult human skeletal muscle. (Zheng B, et al. Manuscript in preparation).
15. Role of PI3K, ERK1/2, and p38 MAPK pathways in BMP4-induced matrix mineralization by MDSCs. (Payne K, et al. Manuscript is in preparation).

Following is the list of current research projects that involve the use of micro-CT core:

1. Guided bone regeneration and bone defect healing using spatial BMP4 protein distribution on prefabricated fibrin and collagen scaffolds using 3D printing technology. (In collaboration with Dr. G. Cooper).
2. Osteogenic potential of human muscle-derived cells on different scaffolds (SIS, fibrin, collagen, etc.) after osteogenic stimulation in vitro and in vivo after transplantation into critical-size calvarial defect (In collaboration with Cook MyoSite, Inc.).
3. Evaluation of skeletal muscle angiogenesis after laceration, contusion or strain injury, electrical stimulation and physical exercise in mice, and its relationship to development of fibrosis (In collaboration with Dr. F. Ambrosio).
4. Evaluation of skeletal muscle vasculature and vertebral bone structure in mouse model of accelerated aging (In collaboration with Dr. L. Niedernhofer and Dr. N. Vo).

Administrative Core
Progress Report
(Johnny Huard, James Cummins and Matthew Bosco)

INTRODUCTION:

The Administrative Core of the Stem Cell Research Center (SCRC) is directly responsible for ensuring the proper function and integration of the Research Laboratories (comprising the Core Research Laboratories, Affiliated Laboratories, and Research Core Facilities), the Clinical Trials Unit, and the Educational Programs that constitute the SCRC. The Administrative Core provides administrative services to all SCRC personnel, supports the ongoing activities of the SCRC, and provides a mechanism for regular evaluation of the SCRC. The Administrative Core also is responsible for fulfilling the secretarial, budgetary, and grant application and manuscript preparation needs of SCRC personnel. In addition, this Core also facilitates collaboration between SCRC researchers and scientists working in designated Collaborative Institutes or other, non- affiliated laboratories.

BODY:

The Administrative Core has provided managerial and financial oversight for the entire Department of Defense Program. The center administrator, Matthew Bosco, and Senior Scientist, James Cummins have played key roles in facilitating and administering all of the various projects within the Program. Mr. Bosco and Mr. Cummins help ensure that each project functions smoothly and efficiently while maintaining continuous communication between the various investigators and institutions.

The Administrative Core organized a weekly/biweekly seminar series for SCRC researchers, affiliates, collaborators, and other interested scientists. The goal of each of these events was to bring in highly regarded scientists who are performing cutting edge research in cellular therapeutics. In addition to serving as a forum in which SCRC scientists can interact with other researchers who share similar interests, this series has helped to introduce the invited speakers to the SCRC and has also helped to promote the development of ongoing collaborations with these well-regarded research scientists. The goal of these meetings is to help SCRC researchers hone their scientific critical analysis skills and, by so doing, improve their own research and writing abilities.

The use of suramin to improve skeletal muscle healing after contusion injury

*Nozaki M, *Li Y, *Zhu J, *Ambrosio F, **Fu FH, +*Huard J
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INTRODUCTION:

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although this type of injury is capable of healing, an incomplete functional recovery often occurs, depending on the severity of the blunt trauma. Complete functional recovery is hindered by the development of scar tissue formation, which typically appears during the second week following muscle injury. We have reported that TGF-β1 is a major factor in triggering the fibrotic cascade within injured skeletal muscle [1]. The use of antifibrosis agents, such as suramin, that inactivates TGF-β1 can reduce muscle fibrosis and consequently improve muscle healing after injury. The ability of suramin to block the fibrotic effect of TGF-β1 and reduce the fibrosis makes this molecule well suited for use in applications to improve muscle healing after injury. We already reported that suramin can effectively prevent muscle fibrosis and enhance muscle regeneration in the lacerated and strain-injured muscle [2,3]; however, it is still not known if suramin can improve muscle healing after contusion injury, which is the most commonly encountered muscle injury. Furthermore it has remained unclear whether this enhanced muscle regeneration is a direct effect of suramin. We performed this study to examine whether suramin would promote differentiation of myogenic cells *in vitro* and improve injured muscle healing by enhancing regeneration and reducing fibrosis *in vivo*, using an animal model of muscle contusion.

MATERIALS AND METHODS:

Muscle-derived stem cell differentiation assay: Muscle-derived stem cells (MDSCs) were isolated from wild type mice(C57BL/6J) via the modified preplate technique [4]. MDSCs (10^4 cells/well) were seeded into 12 well plates and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 10% horse serum, 0.5% chicken embryo extract, and 1% penicillin/streptomycin. After 24 hours, the medium was replaced with differentiation medium (DMEM containing 2% horse serum and 1% penicillin/streptomycin) containing different concentration of suramin (0, 1, 10, 100μl/ml). After another 24 hours, the medium was replaced with only differentiation medium. All cells were grown at 37°C in 5% CO_2. Three days after incubation the fusion index was assessed by counting the number of nuclei in differentiated myotubes as a percentage of the total number of nuclei.

Immunocytochemistry in vitro: Immunocytochemistry was performed on the cells *in vitro* to examine their expression of fast myosin heavy chain (MyHC). Mouse anti-MyHC (1:250; Sigma) was used as the primary antibody, and biotinylated anti-mouse IgG (1:200; Vector) was used as the secondary antibody. Streptavidin 555 conjugate (1:500; Molecular Probes) was applied to detect the secondary antibody.

Animal model: The muscle contusion model was developed in normal wild-type mice (7-10 weeks, average weight 24.0g). A 17g stainless steel ball was dropped through an impactor from a height of 100cm onto the animal's tibialis anterior (TA) muscle. Mice were divided into 4 groups (5mice/group). Different concentrations of suramin (0, 2.5, 5, 10mg in 20 μl of Phosphate-buffered solution [PBS]) were injected intramuscularly two weeks after injury. Cryostat sections of muscles (10μm in thickness) were obtained and histologically stained (hematoxlin and eosin stain (H&E) and Masson's Trichrome stain) four weeks after injury. The numbers of the centronucleated regenerating myofibers from each group was determined to evaluate the regeneration. Northern Eclipse software (Empix Image, Inc.) was used to quantify the total fibrotic area. Statistical analysis was performed with ANOVA.

RESULTS:

Suramin stimulates MDSCs's differentiation: Suramin treatment promoted the differentiation of MDSCs *in vitro* in a dose dependent manner. We observed a significantly higher fusion index in each of the two suramin treatment groups (10 and 100 μg/ml) than in the control group (0μg/ml). Furthermore, 100μg/ml of suramin treatment enhanced the differentiation significantly more than the other suramin treatments (1 and 10μg/ml) (Fig.1,2).

Suramin enhances muscle regeneration and decreases fibrosis after contusion injury: We observed a significant increase in the number of regenerating myofibers in all of suramin treated groups (2.5, 5, 10mg/20μl PBS) when compared with the control group (0mg/20μl of PBS) (Fig. 3,4). Moreover Masson's Trichrome staining showed significantly less fibrotic area in all of suramin treated groups than in the control group (Fig. 5, 6). Although all three suramin treated groups showed significant improvement in healing by way of muscle regeneration and fibrosis inhibition, there was no significant difference between the three suramin treatment groups (data not shown).

Figure 1. Immunocytochemistry of MyHC

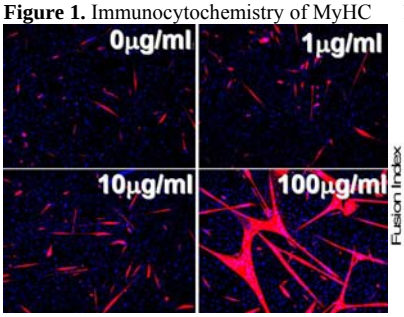


Figure 2. Fusion Index

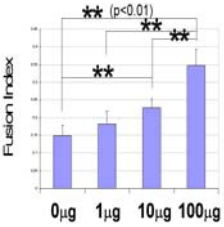


Figure 3. H-E staining 4 wks after contusion

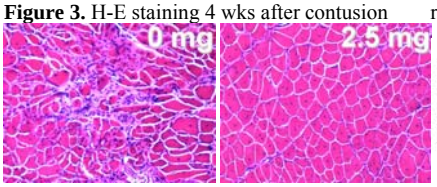


Figure 4. Number of regenerated myofibers

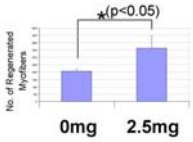


Figure 5. Trichrome staining after contusion

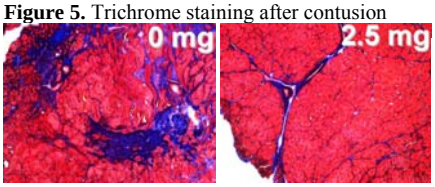
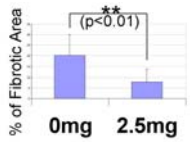


Figure 6. Percentage of Fibrotic area



DISCUSSION:

We have reported that suramin can effectively prevent muscle fibrosis and enhance muscle regeneration after laceration and strain injury. Furthermore, our preliminary data has indicated that suramin blocks the proliferative effect of TGF-β1 on fibroblasts *in vitro* [2,3]. We examined the effect of suramin on the differentiation of MDSCs after we observed that suramin enhanced the differentiation of C2C12 as a preliminary study. Our results from this experiment showed that the suramin treated MDSC groups have higher fusion indices than the control group in vitro. This indicates that suramin can enhance the differentiation of MDSCs, revealing part of the mechanism by which suramin enhances the muscle regeneration after injury. This is the first study to show that suramin is affecting the differentiation of MDSCs directly in addition to it's antiproliferative effect on fibroblasts. Furthermore, our result shows suramin can enhance muscle regeneration and prevent fibrosis after a contusion injury, the most common muscle injury. Our future study will investigate the mechanism by which suramin enhances the differentiation of myogenic cells.

Acknowledgements:

The authors are grateful for technical assistance from Maria Branca, Jessica Tebbets, and Aiping Lu. Funding support was provided by a grant from the Department of Defense (W81XWH-06-1-0406).

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**Department of Orthopaedic Surgery, University of Pittsburgh

Appendix 2

Decorin Gene Transfer Promotes Muscle Cell Differentiation and Muscle Regeneration

Yong Li[1,2,3], Juan Li[2], Jinghong Zhu[1], Bin Sun[1], Maria Branca[1], Ying Tang[1], William Foster[1], Xiao Xiao[2] and Johnny Huard[1,2,4]

[1]*Stem Cell Research Center, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania, USA;* [2]*Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania, USA;* [3]*Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA;* [4]*Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania, USA*

We have shown that decorin, a small leucine-rich proteoglycan, can inhibit transforming growth factor (TGF)-β1 to prevent fibrous scar formation and improve muscle healing after injury. In the decorin-treated muscle, an enhancement of muscle regeneration is observed through histological examination. In this article, we report our determination of whether decorin has a direct effect on myogenic cells' differentiation. Our results indicate that myoblasts genetically engineered to express decorin (CD cells) differentiated into myotubes at a significantly higher rate than did control myoblasts (C2C12). This enhanced differentiation led to the up-regulation of myogenic genes (*Myf5*, *Myf6*, *MyoD*, and myogenin) in CD cells in vitro. We speculate that the higher rate of differentiation exhibited by the CD cells is due to the up-regulation of follistatin, peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α), p21, and the myogenic genes, and the down-regulation of TGF-β1 and myostatin. Decorin gene transfer *in vivo* promoted skeletal muscle regeneration and accelerated muscle healing after injury. These results suggest that decorin not only prevents fibrosis but also improves muscle regeneration and repair.

Received 13 November 2006; accepted 20 May 2007; published online 3 July 2007. doi:10.1038/sj.mt.6300250

INTRODUCTION

Decorin, a small leucine-rich proteoglycan, is a component of the extracellular matrix of all collagen-containing tissues.[1] Decorin is pivotal in regulating the proper assembly of collagenous matrices and in controlling cell proliferation under various conditions.[2] On the basis of its ability to bind fibrillar collagen and delay *in vitro* fibrillogenesis, decorin is regarded as a key modulator of matrix assembly.[3,4] This proteoglycan can modulate the bioactivity of growth factors and act as a direct signaling molecule to different cells.[5] Decorin, which is expressed at high levels in skeletal muscle during early development,[6] also interferes with muscle cell differentiation and migration and regulates connective tissue formation in skeletal muscle.[7–9]

Because terminal differentiation is critical for initial skeletal muscle development and regeneration after injury and disease,[10] we examined decorin's role in remodeling healing skeletal muscle. We have shown that the direct injection of bovine decorin decreased muscle fibrosis and provided nearly complete functional recovery.[11] Decorin blocks fibrosis (mostly by inhibiting transforming growth factor (TGF)-β activity), which improves muscle healing. However, the role of decorin in muscle cell differentiation and regeneration is still unknown. Although we hypothesize that decorin's effect on muscle fibrosis may indirectly impact regeneration, we were unable to exclude the possibility that decorin promotes regeneration independent of its effects on fibrosis formation.

Many studies have investigated the mechanism behind the antifibrotic effect of decorin.[3,12–14] Others have shown that hepatocyte growth factor increases decorin production by fibroblasts through the extracellular signal–regulated kinase 1/2, and p38 mitogen-activated protein kinase–mediated pathways.[14] Decorin stimulates the growth of smooth muscle cells under specific conditions and influences the growth of epidermal cells by interacting with epidermal growth factor and its receptors.[15,16] Recent research has shown that decorin can bind both insulin-like growth factor-I and its receptor; this interaction leads to the phosphorylation of protein kinase B (Akt) and p21 expression in endothelial cells.[17]

Decorin also influences muscle cell behavior by interacting with p21, an important cyclin-dependent kinase inhibitor.[18,19] Follistatin and myostatin are involved in the control of muscle mass during development. These two proteins have opposite effects on muscle growth, as documented by genetic models.[21,22] Recent studies have shown that myostatin action is inhibited by decorin,[23] resulting in enhanced healing and reduced fibrosis within myostatin-null mice compared with wild-type mice.[24] A recent study indicates that peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α), is also involved in the muscle healing process and influences muscle fiber–type determination.[25,26] Decorin may also interact with PGC-1α expression in skeletal muscle after injury.

In this study, we investigated the *in vitro* effect of decorin on the differentiation of myoblasts (C2C12) and characterized the *in vitro* and *in vivo* behavior of myoblasts transfected with the

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decorin gene (CD cells). We also studied the influence that decorin over-expression had on myostatin, follistatin, PGC-1α, and p21 expression. Using an adeno-associated virus (AAV) vector, we transduced the decorin gene into injured skeletal muscle to further investigate its function on muscle healing. Our overall goal in this study was to determine whether decorin could improve skeletal muscle healing by enhancing muscle regeneration independently of its antifibrotic action.

RESULTS

Genetic engineering of myogenic cells to over-express decorin

We used lipofectin to transfect a pAAV-CMV-decorin plasmid (**Figure 1a**) into both 293 cells (packaging cell line) and C2C12 cells (myoblast cell line). The results of western blot analysis (**Figure 1b**) showed decorin in both the supernatant (culture media) and the lysate of the 293 cells 48 hours after transfection. The transfected C2C12 cells (CD clone cells) expressed decorin (**Figure 1c:** lane 2, 24 hours; lane 4, 48 hours; lane 5, decorin-positive control), whereas non-transfected C2C12 cells did not (**Figure 1c:** lane 1, 24 hours; lane 3, 48 hours). We also detected decorin in both myoblasts (C2C12) and muscle-derived stem cells after mDecorin-AAV (mDec-AAV) gene transfer *in vitro* (**Figure 1d** and **e**).

Decorin stimulates myoblast differentiation *in vitro*

To investigate myoblast differentiation, we compared decorin-co-cultured C2C12 cells with non-treated C2C12 cells *in vitro*. C2C12 cells cultured with decorin (10 μg/mL) and grown in differentiation/fusion medium exhibited significantly enhanced differentiation and fusion *in vitro*. After 3 and 4 days of stimulating C2C12 cells with decorin, we observed a significant increase in the number of myotubes when compared with un-stimulated C2C12 (control) cells ($P < 0.01$ at 3 days and $P < 0.05$ at 4 days, respectively). However, the numbers of myotubes 5 days after treatment were not significantly different (**Figure 2a**). We then evaluated whether CD cells exhibited a greater propensity to undergo myogenic differentiation than did non-transfected C2C12 cells. As shown in **Figure 2b** (myotubes stained in mouse anti-myosin heavy chain are red) and **Figure 2c**, CD cells generated significantly more myotubes overall and created significantly larger myotubes than did non-transfected C2C12 cells.

Decorin increases myoblast differentiation and induces myogenic gene expression *in vitro*

We investigated whether the CD cells expressed higher levels of myogenic genes than did non-transfected C2C12 (control) cells. Our results, shown in **Figure 3a**, demonstrate that decorin gene transfer led to higher expression of the myogenic genes *Myf5*, *Myf6*, *MyoD*, and *myogenin*. Desmin expression levels in CD and C2C12 cells remained similar.

Decorin up-regulates p21, follistatin, and PGC-1α, but down-regulates TGF-β1 and myostatin in C2C12

We also performed experiments designed to investigate the mechanism by which decorin influences the differentiation of muscle cells. We found that CD cells exhibited increased p21 expression and decreased myostatin expression (**Figure 3b**). The C2C12 cells can be induced to express TGF-β1 in an autocrine manner, as we have previously determined;[27] however, the CD cells do not show any detectable expression of TGF-β1 after

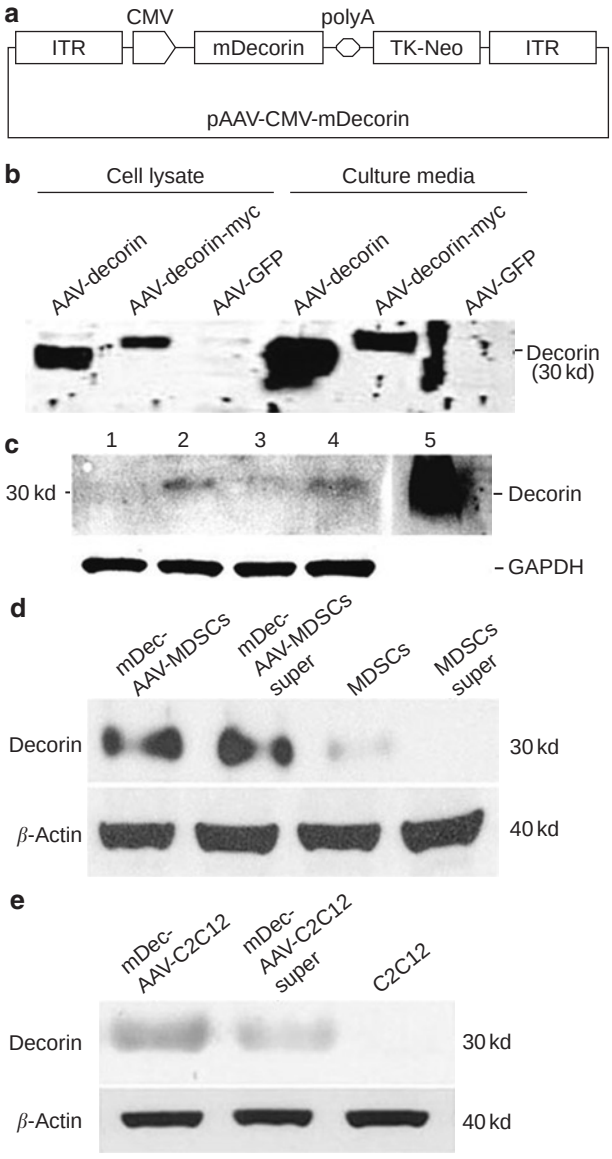

Figure 1 Decorin plasmid construction and initial transfection *in vitro*. (**a**) The decorin plasmid used for the study contained the full sequence of a mouse decorin gene inserted at the *Not*I site, which placed it under the control of a cytomegalovirus (CMV) promoter. (**b**) We transfected the plasmid into 293 cells. We observed decorin expression in both the 293 cells and their supernatant, but not in the control adeno-associated virus (AAV)–transfected (green fluorescent protein, GFP) cells. (**c**) Western blot analysis also revealed decorin expression in CD clone cells within different time period cultures (lane 2, 24 hours; lane 4, 48 hours), but not in C2C12 (lane 1, 24 hours; lane 3, 48 hours). We used 5 μg of decorin as a positive control (lane 5). GAPDH, glyceraldehyde-3-phosphate dehydrogenase is used as a control. (**d**) Decorin expression in muscle-derived stem cells (MDSCs) in pellet form was low, but this was not the case in the supernatant. Both MDSCs and their cultured supernatant strongly expressed decorin after mDec-AAV gene transfer. β-actin is used as a control. (**e**) We did not detect decorin in normal C2C12 cells, but C2C12 cells and their cultured supernatant both expressed decorin after mDec-AAV gene transfer.

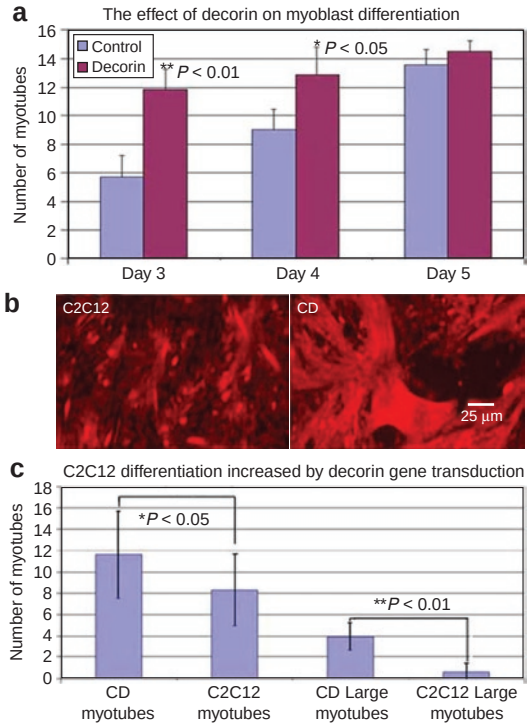

Figure 2 Decorin stimulates C2C12 differentiation *in vitro*. (**a**) Decorin treatment accelerated the differentiation and fusion of myoblasts (C2C12 cells) compared with non-treated myoblasts (C2C12 cells). (**a**) The cultures of decorin-treated C2C12 cells contained more myotubes at the 3- and 4-day time points than control cells. (**b**, **c**) Similarly, decorin-transfected C2C12 clone cells (CD cells) produced more myotubes than did C2C12 cells, including larger myotubes (containing more than three nuclei) *in vitro*. Red staining shows myosin heavy chain fluorescence after immunostaining (**b**).

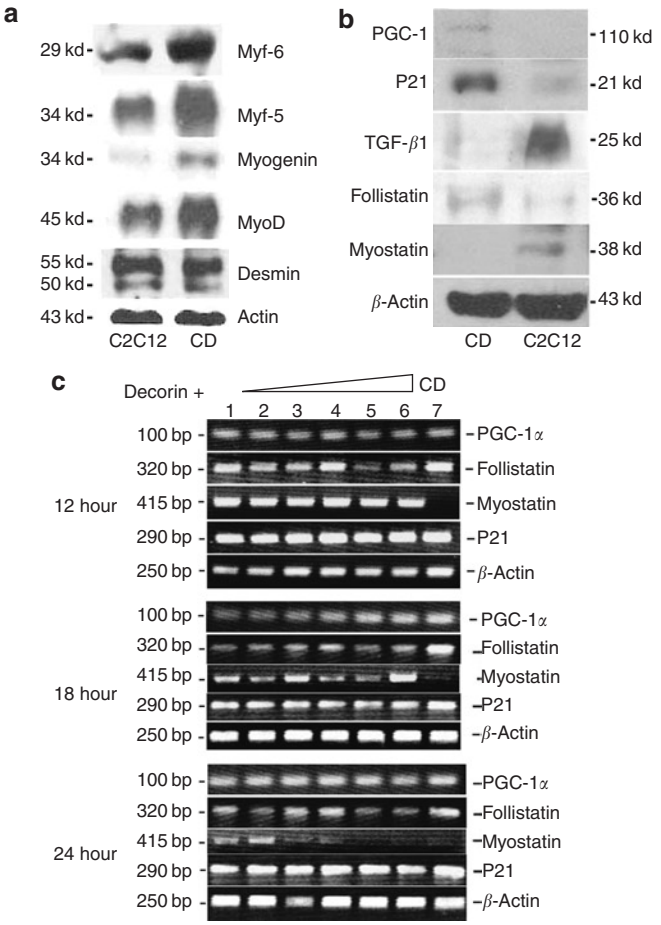

Figure 3 Decorin gene transfer up-regulates myogenic proteins and p21 and down-regulates myostatin during muscle cell differentiation. (**a**) Genetic engineering of myoblasts to express decorin influenced the expression of myogenic proteins (including Myf5/6, Myogenin, and MyoD), as shown by western blot results. However, C2C12 cells and CD clone cells expressed comparable levels of desmin. (**b**) We detected the presence of p21 expressed in CD clone cells. We also detected peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α) and follistatin expressed in CD, but not C2C12, cells. In addition, CD cells exhibited lower levels of myostatin, a negative regulator of muscle mass. The induction of tumor growth factor (TGF)-β1 auto-expression was also inhibited by decorin over-expression in CD cells. (**c**) Similar results were obtained by real-time polymerase chain reaction. Lanes 1–6 show results for C2C12 cells exposed to different concentrations of decorin (0, 0.001, 0.01, 0.1, 1.0, and 5.0 ng/ml, respectively). Lane 7 displays the test results for CD cells, which served as the positive control. We did not detect a visible change in the expression of PGC-1α, follistatin, myostatin, or p21 after 12 hours of stimulation with different concentrations of decorin. PGC-1α and follistatin were up-regulated in C2C12 cells in a dose-dependent manner after 18 hours of stimulation with decorin. Myostatin was down-regulated in C2C12 cells in a dose-dependent manner after 24 hours of decorin stimulation. The concentration of p21 did not visibly change after cell stimulation with any experimental concentration of decorin over all time points. With decorin gene transfer, we found that CD cells consistently expressed follistatin, p21, and PGC-1α but were negative for myostatin. Note that β-actin was selected as a positive gene control.

TGF-β1 stimulation. More importantly, we detected that both follistatin and PGC-1α (**Figure 3b**) had been up-regulated when compared with C2C12 cells. We also discovered that follistatin and PGC-1α messenger RNA were altered in C2C12 cells after decorin stimulation, as determined by real-time polymerase chain reaction. Specifically, we found that PGC-1α and follistatin increased in a dose-dependent manner after 18 hours of stimulation with decorin, and that myostatin was decreased in a dose-dependent manner after 24 hours of stimulation with decorin (**Figure 3c**). In CD cells, we also observed increased amounts of all three genes (*p21*, *follistatin*, and *PGC-1α*), but a decrease in myostatin was observed (**Figure 3c**).

The up-regulation of *follistatin*, *PGC-1α*, *p21*, and myogenic genes, including *MyoD* (**Figure 3a**), in CD cells could at least partially explain how decorin promotes muscle cell differentiation. Alternatively, the down-regulation of myostatin, a well-known negative regulator of muscle growth during muscle regeneration, could also benefit muscle cell differentiation.

The implantation of CD cells in skeletal muscle results in improved muscle regeneration

The implantation of CD cells within skeletal muscle resulted in significantly better muscle regeneration than that observed for control C2C12 cells, as determined 4 weeks after injection of the cells into *MDX*/SCID mice. Although the number of LacZ-positive muscle fibers (*i.e.*, regenerating muscle fibers) did not differ between the groups (**Figure 4a**, **c**, and **d**), the diameters of the regenerating muscle fibers (*e.g.*, dystrophin-positive myofibers) in the muscles injected with CD cells were significantly larger than those of the regenerating muscle fibers in the control muscles

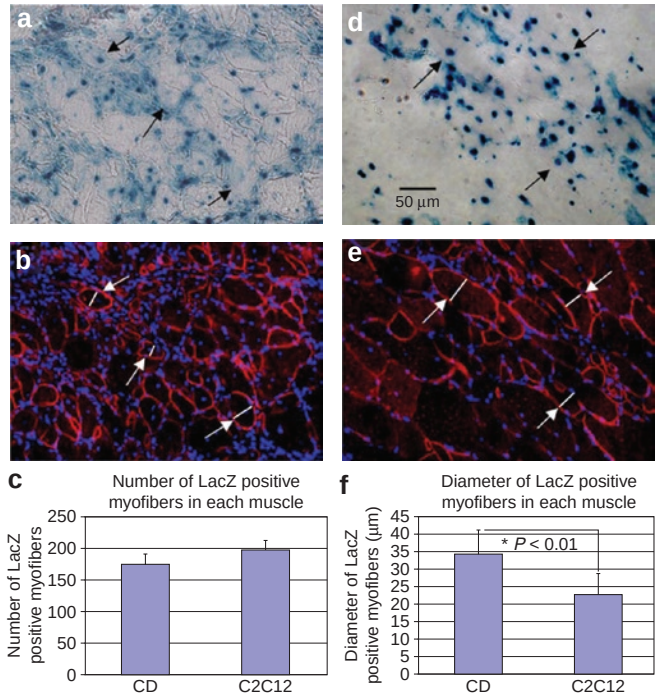

Figure 4 Decorin gene transfer stimulates muscle regeneration *in vivo*. C2C12 cells regenerated muscle fibers after transplantation into skeletal muscle of *MDX*/SCID mice, as shown on both (**a**) LacZ- and (**b**) dystrophin-expressing myofibers. However, transplantation of CD clone cells, rather than C2C12 cells, resulted in larger muscle fibers in *MDX*/SCID mice, as shown in some of the (**d**) LacZ- and (**e**) dystrophin-positive myofibers. (**c**) Although there was no significant difference between the number of LacZ-labeled muscle fibers that formed in muscles transplanted with C2C12 or CD cells, (**f**) the transplantation of CD cells resulted in the regeneration of larger-diameter myofibers ($P < 0.01$).

(**Figure 4b**, **e**, and **f**; dystrophin is red). The larger diameters of the dystrophin-positive muscle fibers generated by CD cells could indicate that implantation of CD cells accelerated muscle regeneration; however, we were unable to exclude the possibility that the CD cells may have a greater propensity to fuse in host myofibers than C2C12 control cells.

mDec-AAV gene transfer promotes muscle regeneration and reduces fibrosis

Better muscle regeneration was observed within mDec-AAV-treated muscle (**Figure 5a** and **b**) than within non-mDec-AAV-treated muscle at 2 weeks after injury (**Figure 5c** and **d**). Histological analysis of total collagen deposition 4 weeks after injury revealed that mDec-AAV-injected muscles contained less fibrous scar tissue in the injured area than did non-treated control muscles (**Figure 5f**, **h**, and **j**; collagen deposition areas are blue). We also observed that decorin stimulated skeletal muscle regeneration 4 weeks after laceration injury. We found that mDec-AAV-injected muscles contained more centronucleated (regenerating) myofibers and less scar tissue 4 weeks after injury than did control muscles (**Figure 5e**, **g**, and **i**).

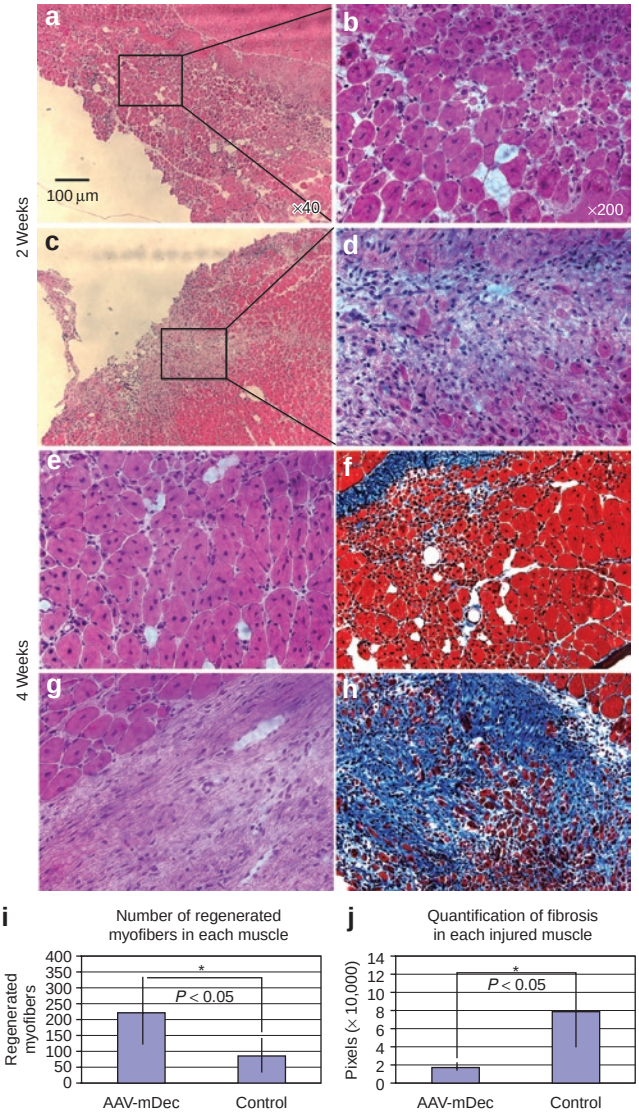

Figure 5 mDec-AAV vector gene therapy in injured muscle prevents fibrosis and promotes muscle regeneration. Decorin-treated muscle exhibits a greater number of regenerating myofibers than the control muscle at all time points (**a–d** 2 weeks; **e–h** 4 weeks). (**i**) The mDec-AAV-injected muscle contained significantly higher numbers of centronucleated (regenerated) myofibers than did control (sham-injected) muscle at 4 weeks after therapy. We also found that decorin gene therapy minimized fibrosis in injured skeletal muscle. We used Masson's trichrome staining to reveal collagen in injured skeletal muscle, the results of which show that (**f**) mDec-AAV-injected muscle contained significantly less fibrosis in the injured area than did the (**h**) control muscle at (**j**) 4 weeks after injury.

DISCUSSION

Results from these experiments show that decorin is able to activate the differentiation of skeletal muscle cells (C2C12) *in vitro* and enhances muscle regeneration in two mouse models *in vivo*. The mechanism behind decorin's accelerated muscle healing is not yet known; however, our results demonstrate that decorin up-regulates the expression of PGC-1α, follistatin, p21, and a variety of myogenic proteins (including MyoD) but down-regulates myostatin expression. These results, in addition to decorin's ability to neutralize the effects of TGF-β1, likely explain the beneficial action that decorin has on muscle cell differentiation and muscle regeneration.

Our previous studies have demonstrated that myogenic cells (including muscle-derived stem cells) in injured muscle can

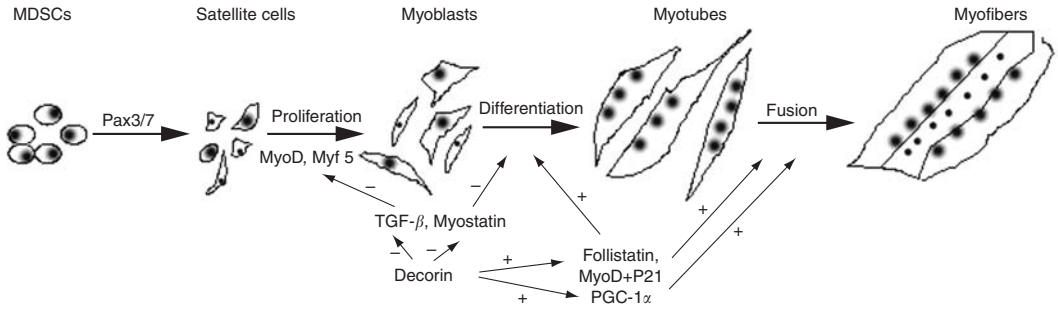

Figure 6 Schematic of the potential effect of decorin on muscle healing. Decorin may improve muscle healing through various pathways: inhibition of tumor growth factor (TGF)-β1, up-regulation of follistatin, peroxisome-proliferator-activated receptor-gamma co-activator-1α (PGC-1α), p21, and myogenic genes (such as *MyoD*), and down-regulation of myostatin expression. MDSCs, muscle-derived stem cells.

differentiate into fibrotic cells and that TGF-β1 is a major stimulator of this differentiation.[27,28] Using different animal models of muscle injury, we have investigated biological approaches to prevent fibrosis and thereby improve muscle healing.[11,27,29–31] We have used various molecules, such as decorin, that impede fibrosis by blocking TGF-β1 to facilitate the near-complete recovery of injured skeletal muscle.[11] The ability of decorin to inhibit TGF-β1 activity is the likely mechanism by which this molecule blocks fibrosis formation. However, our results indicate that the improved muscle healing observed after decorin treatment is due to both its inhibiting effect on fibrosis and its stimulating effect on muscle regeneration. **Figure 6** summarizes the potential effect of decorin on muscle healing.

The repair of injured skeletal muscle occurs through the activation of muscle precursor cells located between the basal lamina and the sarcolemma, including satellite cells and stem cells.[32] The activation and growth of these cells are regulated by various growth factors released by infiltrating lymphocytes, injured myofibers, and the extracellular matrix.[10,32] Some growth factors, such as insulin-like growth factor-1 and hepatocyte growth factor, can stimulate precursor cell proliferation and differentiation by increasing the transcriptional activity of the muscle basic helix–loop–helix.[33–36] Healing and organizational processes are dependent upon the extra- and intracellular signaling that induces the expression of myogenic genes, including *MyoD*, *Myf5*, and *myosin*.[37,38] When properly stimulated, precursor cells fuse with one another or with local myofibers to repair the damaged muscle.[39]

Muscle regeneration, the key event in muscle healing, is often incomplete, particularly in severely injured muscle.[10,11,27,28,32,40] The overgrowth of the extracellular matrix leads to significant local fibrosis (*i.e.*, fibrous scar formation) in the injured area, which can impede the formation of normal muscle fibers. The presence of fibrous scar tissue in injured muscle results in incomplete functional recovery and a propensity for re-injury.[41] Muscle regeneration and fibrosis in injured muscle often occur simultaneously and thus compete with one another during the muscle healing process.[32,36,40] A persistent imbalance between collagen biosynthesis and degradation contributes to hypertrophic scar formation and fibrosis in many tissues.[42,43] Several studies have revealed high levels of collagen in injured regions of skeletal muscle, and shown that inhibition of collagen deposition reduced the formation of scar tissue in injured skeletal muscle.[27,28,30,31]

Interactions between decorin and TGF-β1 have been observed in many tissues, and researchers have used various animal models to study the antifibrotic effect of decorin.[3,11,13,44] Researchers have also shown that hepatocyte growth factor can increase the level of decorin expression in fibroblasts, perhaps by activating the extracellular signal–regulated kinase 1/2 and p38 mitogen-activated protein kinase–mediated pathways.[14] Such findings could explain the antifibrotic effect of hepatocyte growth factor in a variety of tissues.[14,45–47] In endothelial cells, decorin binds with both insulin-like growth factor-I and its receptor to influence cell behavior.[17] Decorin can also control and suppress cancer growth and invasion, presumably by influencing the biological activity of growth factors such as TGF-β1, platelet-derived growth factor, vascular endothelial growth factor, and epidermal growth factor, all of which are released by cancer cells.[5,15] These decorin-induced effects appear to be mediated, at least in part, by a specific interaction between the decorin protein core and the epidermal growth factor receptor.[15,16] This interaction triggers a signal cascade that results in activation of mitogen-activated protein kinase, mobilization of intracellular calcium, up-regulation of p21, and, ultimately, the suppression of tumor growth.[18,19]

Cell cycle exit and the differentiation of muscle cells are coordinated by p21, which is essential for normal myogenic progenitor cell differentiation and skeletal muscle regeneration. Studies have indicated that p21 is necessary for MyoD-induced activity in cells, allowing them to enter into and be stabilized in a postmitotic state. Since MyoD plays a central role in the differentiation of muscle cells, TGF-β1 controls myostatin-related regulation of myogenesis in muscle cells by down-regulating both p21 and MyoD. In this study, we determined that the treatment of myoblasts with decorin down-regulated the expression of myostatin, which might influence p21 and myogenic protein expression. In addition, myostatin and follistatin interact directly in the skeletal muscle system. Follistatin can inhibit myostatin, leading to muscle differentiation in a concentration-dependent manner.[21] PGC-1α, which is expressed in several tissues, including brown fat and the skeletal muscle of mammals, activates mitochondrial biogenesis and oxidative metabolism.[26] PGC-1α is a principal factor involved in determining muscle fiber type in injured skeletal muscle and is involved in exercise-induced mitochondrial biogenesis.[48] In this experiment, we observed that decorin treatment increased PGC-1α expression in skeletal muscle cells. Combined with our previous research results, our current findings suggest that decorin not

only acts as an antifibrotic agent but also enhances muscle regeneration in skeletal muscle.

Successful muscle differentiation during limb development requires decorin expression.[6] Previous findings have shown that decorin can improve muscle healing by inhibiting fibrosis and that myoblasts and muscle satellite cells expressing decorin in an injured site regenerated damaged myofibers faster than the controls.[11,27] The results of this study demonstrate that decorin is also a potent stimulator of skeletal muscle regeneration. Myoblasts expressing decorin differentiated and fused to form myotubes and myofibers at a significantly higher rate than did normal myoblasts *in vitro* and *in vivo*. We attribute this enhanced differentiation to the up-regulation of p21, follistatin, PGC-1α, and myogenic gene expression and the down-regulation of TGF-β1 and myostatin. These results provide at least a partial explanation of the way in which decorin promotes muscle regeneration and may explain why there is such a high level of decorin expression in developing skeletal muscle. It is possible that decorin increases muscle fiber growth and limits the overgrowth of connective tissues. These findings indicate that decorin could be very useful in promoting the healing of muscles damaged by injury or disease.

MATERIALS AND METHODS

Gene transfection and transfer. An AAV-mDecorin plasmid, which encodes for a mouse decorin sequence under the control of the cytomegalovirus promoter (**Figure 1**), was used for gene transfection. This plasmid also contains a neomycin resistance gene to enable G418 selection. The AAV-mDecorin plasmid was transfected into 293 packaging cells and C2C12 cells with lipofectin; clone cells were selected for treatment of the cells with G418 (500 μg/mL) (Gibco BRL, Grand Island, NY) for 2 weeks. The selected decorin-transfected C2C12 clone cells (CD cells) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) containing the same concentration of G418 for the remainder of the project.

The mDec-AAV vector was produced by co-transfection methods described previously by Dr. Xiao.[49] Muscle-derived stem cells and myoblasts (C2C12 cells) were each grown to 50–60% confluency. Fresh Dulbecco's modified Eagle's medium (without fetal bovine serum or penicillin/streptomycin) containing the mDec-AAV vector (5×10^4 particles/cell) was then added directly to the cells. The cultures were incubated at 37 °C in a 5% CO_2 incubator for 1 hour. Normal culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% Abs) was then added for another 24 hours, at which point the cells were collected for analysis of decorin expression by western blotting.

Differentiation of myoblasts and immunocytochemistry. Three different groups of cells (C2C12 cells, C2C12 cells cultured with decorin, and CD cells) were seeded into 12-well plates containing proliferation medium.[28] All cells were transferred into serum-free medium 12 hours later to induce differentiation. The myotubes that formed in the cultures were counted daily for 5 days, and the numbers were compared among the groups. We considered myotubes containing three or more nuclei to be large myotubes *in vitro*. At different time points, the cells were fixed with cold acetone (3 minutes) for immunostaining. Mouse anti-myosin heavy chain antibody (Novocastra Lab) at a 1:200 dilution was applied for 1 hour at room temperature (RT). The primary antibody was detected using anti-mouse-Cy3, 1:250 for 45 minutes at RT. Results were analyzed by fluorescent microscopy (Nikon microscope, Nikon, Melville, New York).

Real-time polymerase chain reaction. Total RNA was extracted from the treated and non-treated C2C12 cells using a Nucleospin column (Clontech, Mountan View, CA), and the complementary DNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), both according to manufacturer's instructions. Primers specific for *myostatin*, *follistatin*, *p21*, and *PGC-1α* were designed using Oligo software (OligoPerfect Designer; Invitrogen, Carlsbad, CA). The protocol for amplification was as follows: 94 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds for 30 cycles. Polymerase chain reaction products were separated by size in a 1.5% agarose gel.

Western blot analysis. C2C12 and CD cells were lysed when cell density reached 70% confluency. The samples were separated on a 12% sodium dodecyl sulfate–polyacrylamide electrophoresis gel and transferred to nitrocellulose membranes used to perform immunostaining. The primary antibodies were anti-decorin (a gift from Dr. Fisher of the National Institutes of Health), anti-TGF-β1 (4 μg/mL; BD Pharmingen, San Diego, CA), anti-p21 (BD Pharmingen, San Diego, CA), anti-myf5, anti-myf6, anti-MyoD, and anti-myogenin (Santa Cruz Bio, Santa Cruz, CA), all at concentrations of 1:1,000, and anti-myostatin, anti-follistatin (Chemicon, Temecula, CA), and anti-desmin (Sigma, St. Louis, MO), all at concentrations of 1:2,000 for 1 hour at RT. Mouse anti-β-actin and anti-glyceraldehyde-3-phosphate dehydrogenase (Sigma, St. Louis, MO) were used for protein quantification and were diluted to 1:8,000. The secondary anti-rat horseradish peroxidase or anti-rabbit horseradish peroxidase (Pierce, Rockford, IL) was used at a concentration of 1:5,000 for 1 hour. Peroxidase activity was determined by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ), and the positive bands were detected on X-ray film. Northern Eclipse software v.6.0 (Empix Imaging, Mississauga, Canada) was used to evaluate all results.

Animal experiments. All animal experiments were approved by the Children's Hospital of Pittsburgh. The Animal Research Committee at the authors' institution approved all experimental protocols (No. 15/03).

Group 1: C2C12 and CD cell transplantation. Twenty-four female *MDX*/SCID mice (C57BL/10ScSn-*Dmd*^{mdx} crossed with C57BL/6J-*Prkdc*^{scid}/SzJ, 6–8 weeks of age) were used for the C2C12 and CD cell transplantation. C2C12 and CD clone cells were transduced with a retrovirus vector encoding for LacZ.[27] LacZ-positive CD cells (1×10^6) were injected into the left gastrocnemius muscles (GMs); the same quantity of LacZ-positive C2C12 cells were injected into the right GMs as a control. At various times after injection, mice were killed, and the GMs were collected for histological analysis by LacZ staining and immunohistochemistry to stain for dystrophin-positive myofibers.

Group 2: mDec-AAV gene therapy administered to injured skeletal muscle. Twenty mice (C57BL6J$^{+/+}$, 6 weeks old; Jackson Laboratory, Bar Harbor, ME) were used for these experiments. The mDec-AAV vector (2×10^{11} particles in 20 μL of Dulbecco's modified Eagle's medium) was injected directly into the left GM of each mouse; the contralateral leg was injected with the same volume of phosphate-buffered saline (20 μL) as a control. One week after injection, both GMs were lacerated in accordance with our previously described muscle injury model.[11,27,28,40] Mice were killed at different time points (5 days and 1, 2, 3, and 4 weeks after injury), and the GMs were collected for histological analysis by either hematoxylin and eosin or Masson's trichrome staining. The regeneration and fibrous scar tissue formation in the two groups were compared.

Immunohistochemical analysis. Serial 10-μm cryostat sections were prepared using standard techniques.[27,28] For immunohistochemistry, the slides were fixed with formalin (4%) for 5 minutes after LacZ staining, and then blocked with donkey serum (10%) for 1 hour. Rabbit anti-dystrophin antibody (Abcam, Cambridge, MA) was applied to the slides at a 1:300 dilution for 60 minutes at RT. The second antibody, goat anti-rabbit IgG (Alexa Fluor® 488; Molecular Probes, Eugene, OR), was used at a concentration of 1:200 for 45 minutes at RT. Negative controls were performed concurrently with all immunohistochemical staining. The nuclei of the sections were revealed using 4′,6′-diamidino-2-phenylindole hydrochloride

staining (Sigma, St. Louis, MO), and fluorescent microscopy was used to visualize the results as described above.

Statistical analysis. LacZ-positive myofibers were counted in 10 representative sections. Both the diameter and number of LacZ- and dystrophin-positive myofibers were assessed at different time points in each group. The statistical significance of differences between the various groups was determined using a *t*-test or one-way or two-way analysis of variance.

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Appendix 3

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Relationships between Transforming Growth Factor-β1, Myostatin, and Decorin

*IMPLICATIONS FOR SKELETAL MUSCLE FIBROSIS**

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Recent studies have shown that myostatin, first identified as a negative regulator of skeletal muscle growth, may also be involved in the formation of fibrosis within skeletal muscle. In this study, we further explored the potential role of myostatin in skeletal muscle fibrosis, as well as its interaction with both transforming growth factor-β1 and decorin. We discovered that myostatin stimulated fibroblast proliferation *in vitro* and induced its differentiation into myofibroblasts. We further found that transforming growth factor-β1 stimulated myostatin expression, and conversely, myostatin stimulated transforming growth factor-β1 secretion in C2C12 myoblasts. Decorin, a small leucine-rich proteoglycan, was found to neutralize the effects of myostatin in both fibroblasts and myoblasts. Moreover, decorin up-regulated the expression of follistatin, an antagonist of myostatin. The results of *in vivo* experiments showed that myostatin knock-out mice developed significantly less fibrosis and displayed better skeletal muscle regeneration when compared with wild-type mice at 2 and 4 weeks following gastrocnemius muscle laceration injury. In wild-type mice, we found that transforming growth factor-β1 and myostatin co-localize in myofibers in the early stages of injury. Recombinant myostatin protein stimulated myofibers to express transforming growth factor-β1 in skeletal muscles at early time points following injection. In summary, these findings define a fibrogenic property of myostatin and suggest the existence of co-regulatory relationships between transforming growth factor-β1, myostatin, and decorin.

Skeletal muscle injuries are one of the most common injuries encountered in sports, accounting for 10–55% of all sports related injuries (1–3). Despite their clinical significance, current treatments remain conservative, such as the RICE principle (rest, ice, compression, and elevation) and non-steroidal anti-inflammatory drugs. However, increasing evidence shows that the administration of non-steroidal anti-inflammatory drugs decreases regeneration and increases fibrosis by inhibiting inflammation (4–8). Although injured skeletal muscle can spontaneously undergo regeneration, muscle regeneration must compete with the ensuing formation of fibrosis, especially in acute injuries (9–11). The resulting excessive fibrotic tissue might form a dense mechanical barrier that prevents the regenerating muscle fibers from maturing (12, 13), thereby resulting in incomplete skeletal muscle healing (14, 15). Researchers have widely accepted that transforming growth factor-β1 (TGF-β1)[3] is a potent stimulator of fibrosis in various tissues (16–19) and is closely associated with skeletal muscle fibrosis as well (20). TGF-β1 levels are elevated in both dystrophic muscles and injured muscles (21, 22). Researchers have also shown that TGF-β1 effectively induces myofibroblastic differentiation of fibroblasts both *in vitro* and *in vivo* (23, 24). The resulting overgrowth of myofibroblasts is responsible for the ensuing excessive accumulation of fibrotic tissue (23, 24). We have previously reported that TGF-β1 plays a significant role in both the initiation of fibrosis and the induction of myofibroblastic differentiation of myogenic cells in injured skeletal muscle (20, 25). Additionally, we have shown that antifibrosis therapies, such as interferon-gamma (INF-γ), suramin, relaxin, and decorin (DCN), improve the healing of injured muscle both histologically and physiologically by blocking the activity of TGF-β1 (26–32). However, it is unclear whether TGF-β1 acts alone or requires interaction with other molecules during the development of muscle fibrosis. Indeed, recent studies have shown that

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[3] The abbreviations used are: TGF-β1, transforming growth factor-β1; MSTN, myostatin; INF, interferon; DCN, decorin; MSTN$^{-/-}$, myostatin knockout; MSTN$^{-/-}$/*mdx* mice, *mdx* mice with myostatin gene knockout; GM, gastrocnemius muscle; PM, proliferation medium; DM, differentiation medium; HS, horse serum; PP1 cells, a population of preplated cells; α-SMA, α-smooth muscle actin; FN, fibronectin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; WT, wide-type; M.O.M., Mouse on Mouse; ECM, extracellular matrix; MRF, muscle regulatory factor; LTP, long-term proliferating; FLST, follistatin; Q-RT-PCR, quantitative reverse transcription-PCR; TβRII, TGF-β1 receptor type II.

myostatin (MSTN), a member of the TGF-β superfamily, may also be involved in fibrosis formation within skeletal muscle (33), although a direct link between MSTN and fibrosis has yet to be identified.

MSTN was initially identified as a negative regulator of muscle development (34), but unlike the ubiquitous expression of TGF-β1, MSTN is predominately expressed in skeletal muscle. MSTN knock-out ($MSTN^{-/-}$) mice, as well as cattle and humans with a naturally occurring MSTN gene mutation, are characterized by a dramatic and widespread increase in skeletal muscle mass (34–36). Interestingly, recent reports suggest that *mdx* mice (an animal model for Duchenne muscular dystrophy) in which expression of the MSTN gene has been ablated ($MSTN^{-/-}$/*mdx*) not only showed better skeletal muscle regeneration but also exhibited decreased fibrosis when compared with *mdx* mice ($MSTN^{+/+}$/*mdx*) (33). These results strongly suggest that MSTN plays an important role in muscle fibrosis. To investigate this possibility, we evaluated the effect of MSTN on fibrosis formation in injured skeletal muscle. Because TGF-β1 plays a major role in the formation of fibrosis, we hypothesized that a relationship between TGF-β1 and MSTN exists. Because DCN has been shown to strongly inhibit fibrosis formation in various tissues via blocking of TGF-β1 activity (26, 27, 37–40), we investigated the potential for DCN to inhibit the activity of MSTN as it does for TGF-β1. Our findings demonstrated that MSTN is involved with fibrosis formation and interacts with TGF-β1 and that DCN has the ability to counteract the action of MSTN. These results contribute to a better understanding of the mechanism of skeletal muscle healing and indicate that MSTN represents a potential pharmacological target for anti-fibrogenic therapy.

EXPERIMENTAL PROCEDURES

Isolation of Fibroblasts from Skeletal Muscle—The preplate technique was used to isolate fibroblasts from skeletal muscle (41). Collagen-coated flasks were used in the isolation process, because fibroblasts adhere more readily to collagen than myoblasts. After 6-week-old female C57BL/6J mice were sacrificed, their gastrocnemius muscles (GMs) were removed and minced into a coarse slurry. The muscle slurry was digested with 0.2% collagenase (type XI) for 1 h, followed by a dispase digestion (grade II, 240 ml) for 30 min, followed by a 0.1% trypsin digestion for a final 30 min at 37 °C. The extracted muscle cells were resuspended in proliferation medium (PM) consisting of Dulbecco's modified Eagle's medium (Invitrogen), 10% horse serum (HS, Invitrogen), 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 0.5% chicken embryo extract (Accurate Chemical & Scientific Corp., Westbury, NY) and plated onto collagen-coated flasks. A population of preplated cells (PP1), consisting of mostly fibroblasts that attached within the first 2 h, was collected and used, in these experiments, as skeletal muscle-derived fibroblasts. This preplate technique was also used to isolate long-term proliferating (LTP) cells (muscle-derived stem cell-like cells) from WT and $MSTN^{-/-}$ muscle (41). Two hours after the initial plating, most of the rapidly adhering fibroblasts attached; the remaining nonadherent cells were transferred to a new collagen-coated flask every 24 h. As this process was repeated, the subsequent populations of late-adhering cells were identified as PP2, PP3, PP4, and PP5 in sequence. Following the collection of PP5, the rest of the cell suspension was incubated for an additional 72 h to allow the cells to attach in another collagen-coated flask. The final adherent cells are LTP cells (41).

Cell Culture—The NIH3T3 fibroblast cell line and the C2C12 myoblast cell line were purchased from the American Type Culture Collection (Manassas, VA). The cell lines or isolated PP1 fibroblasts were maintained in PM consisting of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 1% penicillin/streptomycin until further needed. PP1 fibroblasts were plated onto collagen-coated 96-well plates for cell-proliferation analysis and onto 6-well plates for the evaluation of α-smooth muscle actin (α-SMA), fibronectin (FN), collagen (types Iα1, IIα2, and IIIα1), and MSTN expression. Following an overnight incubation, PM was replaced with serum-free medium supplemented with a serum replacement (Sigma) consisting of heat-treated bovine serum albumin, heat-treated bovine transferrin, and bovine insulin. This serum replacement does not contain growth factors, steroid hormones, glucocorticoids, or cell adhesion factors. We further supplemented this media with varying concentrations of recombinant human MSTN (Leinco Technologies, Inc., St. Louis, MO) for proliferation assays (0, 100, 500, or 1000 ng/ml) and for Western blot analysis (0, 100, or 500 ng/ml). After incubation for 48 h, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay kit (Roche Diagnostics, Germany) was used to measure cell proliferation ($n = 6$) following the instructions from the manufacturer. Western blot analysis was used to examine α-SMA, FN, and MSTN expression. Some of the above procedures were repeated using NIH3T3 fibroblasts to confirm the effect of MSTN on fibroblasts.

C2C12 myoblasts, a widely used myogenic cell line (42–44), were used to examine whether DCN neutralized the inhibitory effect of MSTN on cell differentiation. We seeded C2C12 myoblasts in 12-well plates in PM at a density of 10,000 cells/well. Following an overnight incubation, PM was replaced with fresh differentiation medium (DM) containing Dulbecco's modified Eagle's medium, 2% HS, and 1% penicillin/streptomycin. We maintained a total of four sets of cultured cells. The control set received only DM, whereas the other sets received DCN alone or 1 μg/ml MSTN combined with 0–50 μg/ml DCN ($n = 3$). Cells were cultured for 5 more days during which DM, MSTN, and DCN were changed every other day. Following a similar procedure, we examined whether recombinant follistatin (FLST) protein stimulated myogenic differentiation of C2C12 myoblasts ($n = 3$), and whether soluble TGF-β1 receptor type II (TβRII, 100 and 1000 ng/ml, R&D Systems, Inc., Minneapolis, MN) was able to attenuate MSTN-inhibited myoblast differentiation ($n = 3$).

Western Blot Analysis—After culturing, the cells were lysed with T-PER® Tissue Protein Extraction Reagent with the addition of protease inhibitors (Pierce). Equal amounts of cellular protein were loaded into each well and separated by 10% SDS-PAGE. Nitrocellulose membrane blotting was performed under standard conditions. The following primary antibodies were used for immunoblotting: mouse anti-β-actin IgG (1:8000, Sigma), mouse anti-glyceraldehyde-3-phosphate dehydrogen-

TABLE 1
Sequence of primer set

Gene name (GenBank™ no.)	Primer pair (S: sense primer, A: anti-sense primer)	PCR products
		bp
Procollagen type Iα1 (BC050014)	S: 5′-GAAGAACTGGACTGTCCCAAC-3′ A: 5′-CCTCGACTCCTACATCTTCTG-3′	103
Procollagen type Iα2 (AK075707)	S: 5′-TCTGGTAAAGAAGGCCCTGTG-3′ A: 5′-GTCCAGGGAATCCGATGTTG-3′	106
Procollagen type IIIα1 (AK041115)	S: 5′-AGGCTGAAGGAAACAGCAAA-3′ (45) A: 5′-TAGTCTCATTGCCTTGCGTG-3′	116
TGF-β1 (BC 013738)	S: 5′-CTAATGGTGGACCGCAACAAC-3′ A: 5′-CACTGCTTCCCGAATGTCTGA-3′	99
18 S rRNA (?)[a]		N/A

[a] Sequences of the primer pairs for 18 S rRNA not provided by Applied Biosystems Inc. for proprietary reasons.

ase IgG (1:5000, Abcam Inc., Cambridge, MA), rabbit anti-MSTN IgG (1:3000, Chemicon, Temecula, CA), mouse anti-α-SMA IgG (1:1000, Sigma), mouse anti-FN IgG (1:3000), and rat anti-TGF-β1 IgG (1:1000, BD Pharmingen, San Jose, CA).

Quantitative RT-RCR—Quantitative RT-PCR (Q-RT-PCR) was used to examine the mRNA expression levels of procollagen (types Iα1, Iα2, and IIIα1) in PP1 fibroblasts treated with MSTN (100, 200, and 500 ng/ml) for 12, 24, and 48 h. The mRNA was extracted using an RNeasy Plus kit (Qiagen). The cDNA templates for Q-RT-PCR were synthesized using a RETROscript® kit (Ambion Inc., Austin, TX). Q-RT-PCR was carried out in an ABI Prism 7000 sequence detector (Applied Biosystems Inc., Foster City, CA) with SYBR Green PCR Master Mix Reagent (Applied Biosystems) as a detector. All target gene expressions were normalized to 18 S rRNA levels. The primer pair of procollagen IIIα1 was from a previous study (45). The primer pairs are displayed in Table 1.

ELISA—Enzyme-linked immunosorbent assay (ELISA) was performed to determine whether recombinant MSTN protein stimulated TGF-β1 secretion in C2C12 myoblasts. C2C12 myoblasts were plated into a 48-well plate and exposed to a range of MSTN concentrations from 0 to 500 ng/ml. Fresh, recombinant MSTN protein was added every 2 days. Cell supernatants were collected at 2 and 4 days ($n = 5$). These supernatants were centrifuged to remove cell debris and stored at −80 °C until the ELISA was performed. The mouse/rat/porcine TGF-β1 immunoassay kit (R&D Systems, Inc.) was used to quantitatively measure the secreted TGF-β1 levels in cell culture supernatants, according to the manufacturer's protocol.

Immunocytochemistry—To monitor the differentiation capacity of the myogenic cells, they were fixed in cold methanol for 2 min after induction of differentiation in 12-well plates. Following a phosphate-buffered saline (PBS) wash, the cells were blocked with 10% HS (Vector Laboratories, Inc., Burlingame, CA) for 30 min, and then incubated with an anti-myosin heavy chain antibody (Sigma) in 2% HS overnight. A negative control was performed by omitting the primary antibody. The next day, after several PBS rinses, the cells were incubated with the secondary antibody goat anti-mouse IgG conjugated with Cy3 (Sigma) for 1 h. Hoechst 33258 dye was used in each experiment to stain cell nuclei. Fusion index (ratio of nuclei in myotubes to all nuclei) was calculated (%) to evaluate myogenic differentiation.

Animal Model—All experimental animal protocols were approved by the Animal Research and Care Committee at Children's Hospital of Pittsburgh (protocols 15-3 and 17-05). C57BL/6 wild-type (WT) (Jackson Laboratories, Bar Harbor, ME) and $MSTN^{-/-}$ mice (7–8 weeks of age) were used in this study. All $MSTN^{-/-}$ mice used were offspring of $MSTN^{-/-}$ homozygotes, and PCR was used to confirm the genotype of all $MSTN^{-/-}$ mice. The RT-PCR test was randomly used to confirm the lack of MSTN gene transcription in $MSTN^{-/-}$ mice throughout the experiments. The skeletal muscle mass of $MSTN^{-/-}$ mice and WT mice were also compared to confirm the desired phenotype. The mice were anesthetized with isoflurane controlled under an IMPAC6 anesthetic delivery machine (VetEquip, Pleasanton, CA). Both GMs of each mouse were laterally lacerated to create an injury model as previously described (27–29). A surgical blade (no. 11) was used to make a lateral laceration through 50% of the muscle width and 100% of the muscle thickness in the area of the GM with the largest diameter. We harvested the mouse GMs at 2 and 4 weeks post-surgery. There were 6–8 mice (12–16 GMs) in the WT and $MSTN^{-/-}$ mouse groups for both time points. The muscles were isolated, removed, and snap-frozen in 2-methylbutane pre-cooled in liquid nitrogen. After Masson's trichrome staining (IMEB Inc., Chicago, IL), Northern Eclipse software (Empix Imaging, Inc., Cheektawaga, NY) was used to measure areas of fibrotic tissue in the injured sites. In each sample, three representative non-adjacent sections were chosen. The ratio of the fibrotic area to the cross-sectional area was used to estimate the extent of fibrosis formation. To determine the skeletal muscle's regeneration efficiency, minor axis diameters (the smallest diameter) of regenerating muscle fibers were measured using Northern Eclipse software on cross-sections of GMs. The diameters of over 350 consecutively centro-nucleated myofibers were measured in each GM.

To analyze the expression of MSTN in the injured GM, 18 8-week-old female C57BL/6 WT mice underwent bilateral GM laceration. Mice were sacrificed at 1, 3, 5, 7, 10, 14, 21, and 30 days after injury ($n = 3$ for each time point), and GMs were harvested, frozen, and stored at −80 °C.

300,000 LTP cells obtained from $MSTN^{-/-}$ mice were transplanted in the GMs of 3 8-week-old *mdx*/scid mice using a protocol previously described (41). The same amount of cells obtained from WT mice was injected into contralateral GMs of *mdx*/scid mice to serve as our control. Mice were sacrificed after 4 weeks, and GMs were frozen in liquid nitrogen. Immunostaining with anti-mouse dystrophin antibody (Abcam Inc.) was performed to detect dystrophin-positive myofibers that regenerated from transplanted cells.

To examine whether the injection of MSTN induced TGF-β1 expression, we injected MSTN (1000 ng in 10 μl of PBS) into the non-injured GM of WT mice. Contralateral GMs were injected with 10 μl of PBS and served as a control. Three WT mice were used at each time point. Mice injected with MSTN were sacrificed at 4, 10, 24, and 48 h after injection ($n = 3$ for each time point). Immunohistochemical staining was performed to detect MSTN and TGF-β1 expression in muscle fibers.

Immunohistochemistry—Frozen GMs were sectioned at 10-μm thickness, and immunohistochemical analysis was performed to detect MSTN and TGF-β1 expression. Tissue sections were fixed in 4% formalin for 5 min followed by two 10-min washes with PBS. The sections were then blocked with 10% HS for 1 h. The rabbit MSTN primary antibody was diluted 1:100 in 2% HS and incubated with sections overnight at 4 °C. The following day, the sections were washed three times with PBS and then incubated with the secondary antibody, goat anti-rabbit IgG conjugated with Cy3 (Sigma). The Mouse-on-Mouse immunodetection kit (M.O.M., Vector Laboratories, Inc.) was then used to stain for TGF-β1 following the manufacturer's protocol. The slides were incubated with M.O.M. blocking reagent for 1 h, washed with PBS, and then incubated with M.O.M. diluent for 5 min. TGF-β1-specific primary antibodies (Vector Laboratories, Inc.) were diluted 1:150 in the M.O.M. diluent and incubated with the slides for 30 min. After washing with PBS, the sections were incubated with anti-mouse IgG conjugated with fluorescein isothiocyanate (diluted 1:200 with M.O.M. diluent, Sigma) for 1 h. Hoechst 33258 dye was used to stain the nuclei. In a separate experiment following a similar procedure, polyclonal rabbit anti-DCN IgG (LF-113, National Institute of Dental Research, Bethesda, MD) was used to stain tissue sections of WT and $MSTN^{-/-}$ GMs 2 weeks after laceration.

Statistical Analysis—All of the results from this study are expressed as the mean ± S.D. The differences between means were considered statistically significant if $p < 0.05$. The Student's *t* test was used to compare the difference in skeletal muscle regeneration, fibrosis formation between $MSTN^{-/-}$ and WT mice, and the myogenic differentiation capacity between $MSTN^{-/-}$ and WT LTP cells. All other data were analyzed by analysis of variance followed by post hoc Tukey's multiple comparison test. *Error bars* on the figures represent the ±S.D. (*, $p < 0.05$; **, $p < 0.01$).

RESULTS

Effects of MSTN on Fibroblasts—MTT proliferation tests showed that, after 48 h of incubation, MSTN significantly stimulated the proliferation of PP1 and NIH3T3 fibroblasts in a dose-dependent manner (Fig. 1*A*). α-SMA, the actin isoform originally found in contractile vascular smooth muscle cells, has been the most reliable marker of myofibroblasts to date (24). Western blot analysis indicated that MSTN (100 and 200 ng/ml) increased α-SMA expression in PP1 and NIH3T3 fibroblasts (Fig. 1*B*). Q-RT-PCR revealed that MSTN stimulated procollagen (type Iα1, Iα2, and IIIα1) mRNA expression at 48 h (Fig. 1*C*). Additionally, MSTN stimulated the expression of FN protein, a component of the extracellular matrix (ECM), in PP1 fibroblasts (Fig. 1*D*).

MSTN Expression in Injured Skeletal Muscle—After laceration injury, different time points were selected to detect MSTN expression in GMs. The degenerative and repair remodeling phases were represented by post-injury time points of 1–3 and 5–30 days following injury, respectively. Immunostaining for MSTN indicated MSTN expression within degenerative myofibers at 1 and 3 days after the injury (data not shown). On day 5, by the time a majority of newly regenerating myofibers was seen, faint MSTN signals were detected in the cytoplasm of regenerating centro-nucleated myofibers (*red fluorescence* and *white arrowheads*), whereas green collagen IV immunostaining indicates basal lamina of myofibers (Fig. 2*A*). MSTN expression was also observed in the nuclei of both the mononuclear cells (*white arrows*) and the regenerating centro-nucleated myofibers (Fig. 2*A*), which is especially obvious in the enlarged image (*white arrowhead* Fig. 2*A*, *inset*). On day 7 (Fig. 2*B*), a decrease in MSTN expression within most of the regenerating myofiber cytoplasm was seen (*white arrowheads*), whereas some myotubes without intact basal lamina were strongly stained with MSTN antibody, which is increased 14 days post-injury (*white arrows*, Fig. 2*C*). The nuclei of myofibers remained MSTN-positive (*yellow arrowhead*, Fig. 2, *B* and *C*, *insets*). MSTN staining disappeared from most regenerated myofibers 30 days after laceration (*white arrowheads*, Fig. 2*D*). Fig. 2, *E*, *F*, *G*, and *H*, depict negative controls of injured muscle at 5, 7, 14, and 30 days after laceration, respectively, where the MSTN antibody was replaced by the non-immune rabbit IgG. Collagen type IV was also stained on these samples to visualize the basal lamina.

Reduced Fibrosis and Enhanced Skeletal Muscle Regeneration in $MSTN^{-/-}$ Mice after Laceration—At 2 weeks following injury, we observed extensive deposition of collagenous tissue in the WT and $MSTN^{-/-}$ mice (data not shown). After 4 weeks, the deepest area of the injured site was filled with regenerating myofibers of large diameter, and the fibrotic region was limited to the superficial zone of the laceration site (Fig. 3*A*). We observed fewer fibrotic connective tissue deposits between regenerating myofibers in the injured muscle of $MSTN^{-/-}$ mice compared with the prominent scar region in the injured WT mouse muscle (Fig. 3*A*). Quantification of fibrotic tissue (*i.e.* the ratio of the fibrotic area to the cross-sectional area) revealed that there was a significantly smaller fibrous area in $MSTN^{-/-}$ skeletal muscle as compared with WT skeletal muscle at 2 weeks (11.5 ± 3.5% *versus* 15.3 ± 3.1%; $p < 0.01$) and at 4 weeks (2.1 ± 0.4 *versus* 6.3 ± 2.1; $p < 0.01$) after injury (Fig. 3*B*).

We used the minor axis diameter (smallest diameter) of centro-nucleated regenerating myofibers to evaluate skeletal muscle regeneration after laceration injury. At 2 weeks after GM laceration, regenerating myofibers were relatively small (data not shown). At 4 weeks, some large, mature myofibers could be observed among the small, centro-nucleated, regenerating myofibers (Fig. 3*C*). Quantification showed that $MSTN^{-/-}$-regenerating myofibers had diameters 38.8% larger than WT myofibers (36.1 ± 2.5 μm *versus* 26.0 ± 2.2 μm, $p < 0.01$) at 2 weeks after laceration, and the mean diameter of regenerating myofibers in $MSTN^{-/-}$ mice remained 21.1% larger than the

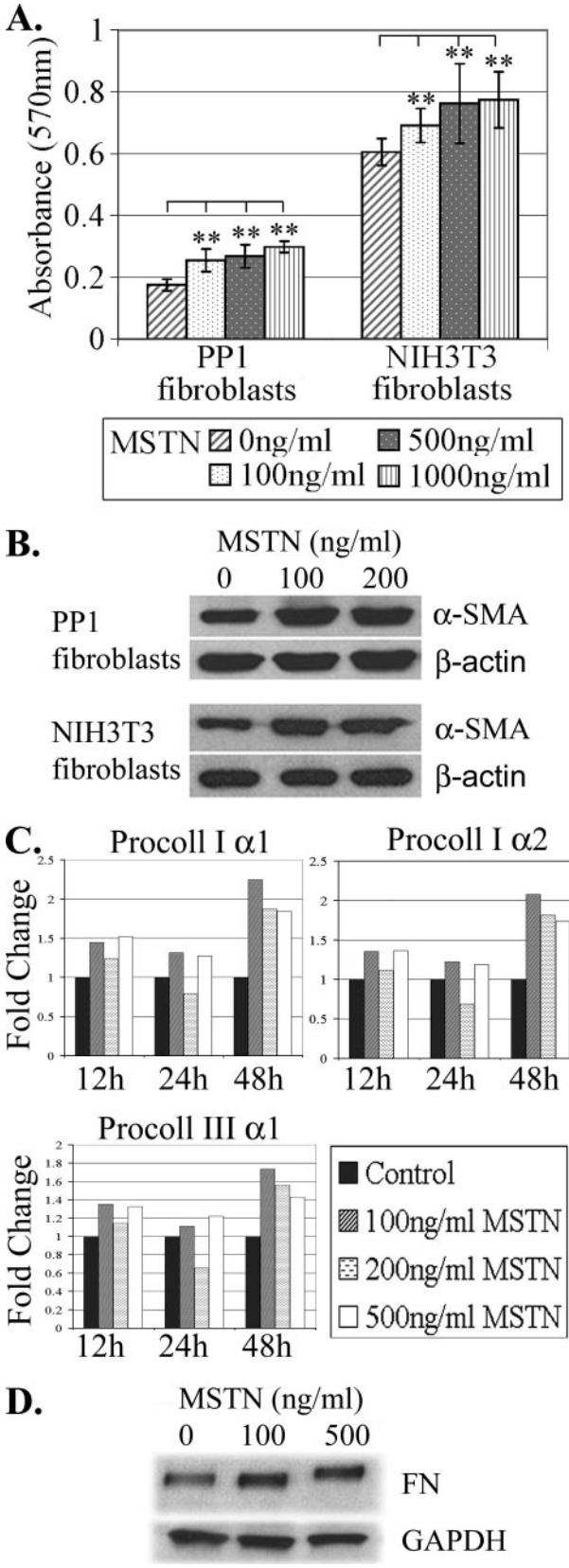

FIGURE 1. **MSTN stimulated fibroblast proliferation and fibrotic protein expression in fibroblasts.** *A*, both muscle-derived fibroblasts (*PP1*) and NIH3T3 fibroblasts were cultured with MSTN, varying in concentration from 0 to 1000 ng/ml for 48 h. Cell proliferation was determined by MTT assay. These results are presented as absorbance values ($n = 6$) of purple formazan crystal at 570 nm, which directly correlates to the number of living cells. Fibroblasts were cultured in DM for 2 days with the addition of various concentrations of MSTN. Expressions of different proteins were analyzed by Western blot. *B*, the expression of α-SMA in PP1 fibroblasts or NIH3T3 fibroblast is shown. *C*, Q-RT-PCR analysis of procollagen (types Iα1, Iα2, and IIIα1) mRNA expression in PP1 fibroblasts treated with MSTN. Results are presented as the ratio against the gene expression in the control. *D*, expression of FN in PP1 fibroblasts after MSTN treatment (*, $p < 0.05$; **, $p < 0.01$).

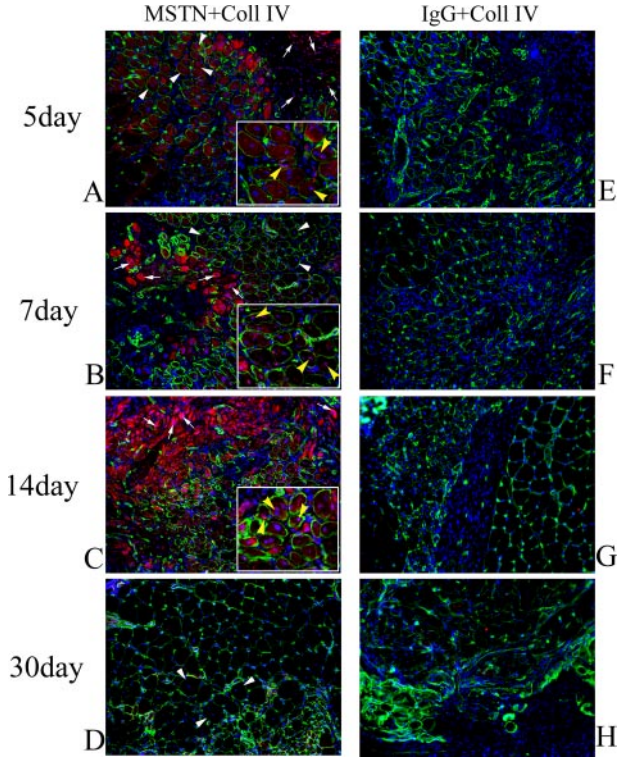

FIGURE 2. **MSTN localization in injured GMs.** *A–H*, GMs from WT mice were harvested at different time points after laceration injury, and frozen sections were immunostained with rabbit anti-MSTN and goat anti-collagen type IV antibodies. MSTN, collagen IV, and cell nuclei are *red*, *green*, and *blue*, respectively (*A–D*). Non-immune rabbit IgG was used as negative control for rabbit anti-MSTN antibody (*E–H*), but were stained with the collagen type IV antibody. *A*, at 5 days, faint MSTN signals could be detected in the cytoplasm of newly formed myofibers (*white arrowheads*) with basal lamina, and a relatively higher MSTN staining can be observed in the nuclei of regenerating myofiber (*yellow arrowhead*) and mononuclear cells (*white arrow*). *B*, at 7 days, MSTN staining is not evident in the cytoplasm of most regenerating myofibers (*white arrowhead*), whereas some of the regenerating small myotubes without basal lamina show intense MSTN staining in the cytoplasm (*white arrows*). *Yellow arrowheads* in the *inset* indicate positive signal in nuclei of regenerating myofibers. *C*, at 14 days, there were more MSTN-positive myotubes without basal lamina (*white arrow*). *Yellow arrowheads* in the *inset* indicate positive signal in nuclei of regenerating myofibers. *D*, at 30 days, most of regenerating myofibers were MSTN-negative (*arrowheads*). (Magnification, ×200; *inset* magnification, ×400.)

mean diameter of regenerating myofibers in the WT mice (37.7 ± 2.7 μm *versus* 31.1 ± 1.8 μm, $p < 0.01$) 4 weeks after injury (Fig. 3*D*). The distribution of the regenerating myofiber diameters showed that there was an increase in the percentage of larger regenerating myofibers in MSTN$^{-/-}$ mice compared with WT mice (*e.g.* ~7.38% of regenerating myofiber diameters in MSTN$^{-/-}$ mice fell into a range of 50–55 μm *versus* 1.92% of those in WT mice).

Improved Myogenic Potential with MSTN$^{-/-}$ LTP Cells—LTP cells were isolated from WT and MSTN$^{-/-}$ mice. When we cultivated these MSTN$^{-/-}$ LTP cells in low serum medium, they differentiated into myotubes that were significantly larger

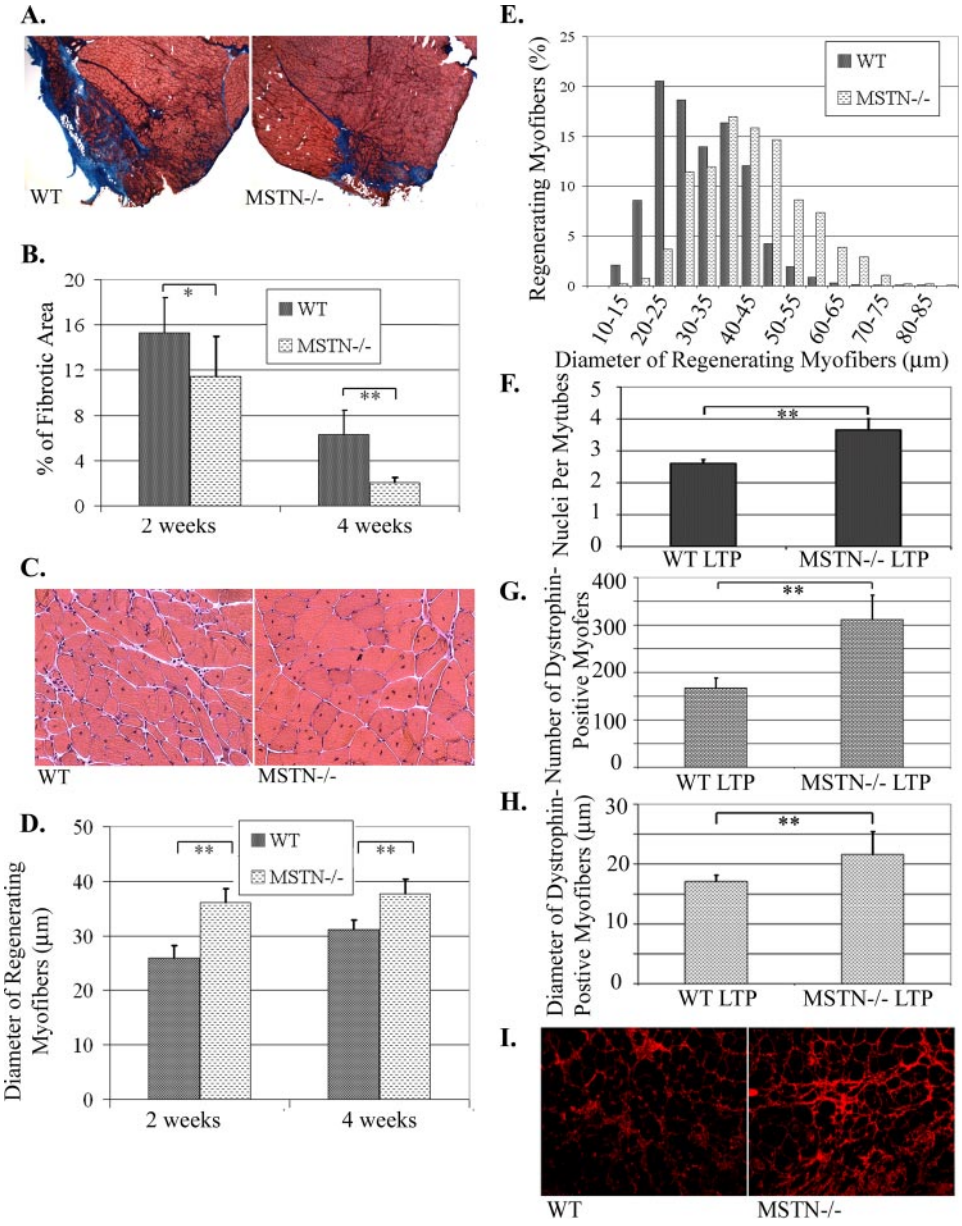

FIGURE 3. **Inhibition of MSTN favors skeletal muscle regeneration.** *A*, sections from injured WT and MSTN$^{-/-}$ GMs were stained with Masson's trichrome-staining protocol 4 weeks after laceration to determine fibrotic tissue levels. As a result, collagenous tissue is stained *blue*. *B*, quantification of fibrotic tissue of WT *versus* MSTN$^{-/-}$ GMs 2 and 4 weeks after laceration. *C*, myofibers in WT and MSTN$^{-/-}$ GMs were visualized by hematoxylin and eosin staining 4 weeks after laceration. Regenerating myofibers were distinguished by their centralized nuclei. *D*, quantification of the diameters of regenerating myofibers. *E*, the distribution of regenerating myofiber diameters at 4 weeks after laceration injury. *F*, myogenic differentiation capacity of WT and MSTN$^{-/-}$ LTP cell *in vitro*. *In vivo*, transplantation of MSTN$^{-/-}$ LTP into *mdx*/scid mice led to a high number of dystrophin-positive muscle fibers when compared with WT LTP. *G*, the number of dystrophin-positive myofibers was counted. *H*, the diameter of dystrophin-positive myofibers was measured. *I*, increased DCN immunostaining in injured skeletal muscle of MSTN$^{-/-}$ mice compared with WT mice 2 weeks after laceration. DCN (*red*) is detected in the ECM between myofibers. (Magnifications: in *C* and *I*, ×200; in *A*, ×100; *, $p < 0.05$; **, $p < 0.01$.)

(more nuclei per myotube, $n = 3$) than the myotubes formed by the fusion of WT LTP cells (Fig. 3*F*). When we injected the MSTN$^{-/-}$ LTP cells into the muscle of *mdx*/scid mice, they regenerated significantly more dystrophin-positive muscle fibers than did the WT LTP (Fig. 3*G*). These regenerating muscle fibers were also significantly larger in diameter (Fig. 3*H*).

Elevated DCN Expression in Injured MSTN$^{-/-}$ Mice—To investigate the underlying mechanism for improved muscle healing in MSTN$^{-/-}$ mice, we examined the expression of DCN, a molecule that has been shown to decrease fibrosis and enhance muscle regeneration (20, 27) in injured MSTN$^{-/-}$ skeletal muscle. Immunohistochemical staining revealed that there was more abundant DCN expression in the regenerating skeletal muscle of MSTN$^{-/-}$ mice than that of WT mice 2 weeks after injury (Fig. 3*I*). This higher level of DCN expression may be involved with the increased regeneration and decreased fibrosis observed in the injured muscle of MSTN$^{-/-}$ mice.

Relationship between TGF-β1 and MSTN$^{-/-}$—Western blot analysis showed that the levels of MSTN in C2C12 myoblasts treated with different concentrations of TGF-β1 were elevated in a dose-dependent manner when compared with non-treated controls, suggesting that TGF-β1 stimulates MSTN expression in C2C12 myoblasts (Fig. 4*A*). After incubation with increasing concentrations of recombinant MSTN protein, MSTN was shown to stimulate TGF-β1 expression in C2C12 myoblasts (especially with the highest dose) at 4 days post-stimulation (Fig. 4*B*). Furthermore, ELISA showed that MSTN significantly increased TGF-β1 secretion by C2C12 myoblasts in a dose-dependent manner at 2 and 4 days. After 4 days of stimulation with MSTN (500 ng/ml), C2C12 myoblasts secreted ~2-fold more TGF-β1 as compared with control cells (Fig. 4*C*). Q-RT-PCR revealed that MSTN (100, 200, and 500 ng/ml) also increased TGF-β1 mRNA expression 48 h post-stimulation (Fig. 4*D*).

PP1 fibroblasts did not express detectable MSTN protein. However, after treatment with MSTN (100 and 200 ng/ml) for 48 h, PP1 fibroblasts began to express MSTN as indicated by Western blot analysis (Fig. 4*E*). MSTN also stimulated MSTN expression in C2C12 myoblasts (Fig. 4*E*). MSTN-induced MSTN autocrine expression in PP1 fibroblasts is reduced by soluble TβRII, which blocks the TGF-β1 signaling pathway (Fig. 4*F*). Moreover, our results indicated that soluble TβRII was also able to restore MSTN-inhibited C2C12 myoblast differentiation (Fig. 4*G*). We also examined whether exogenous

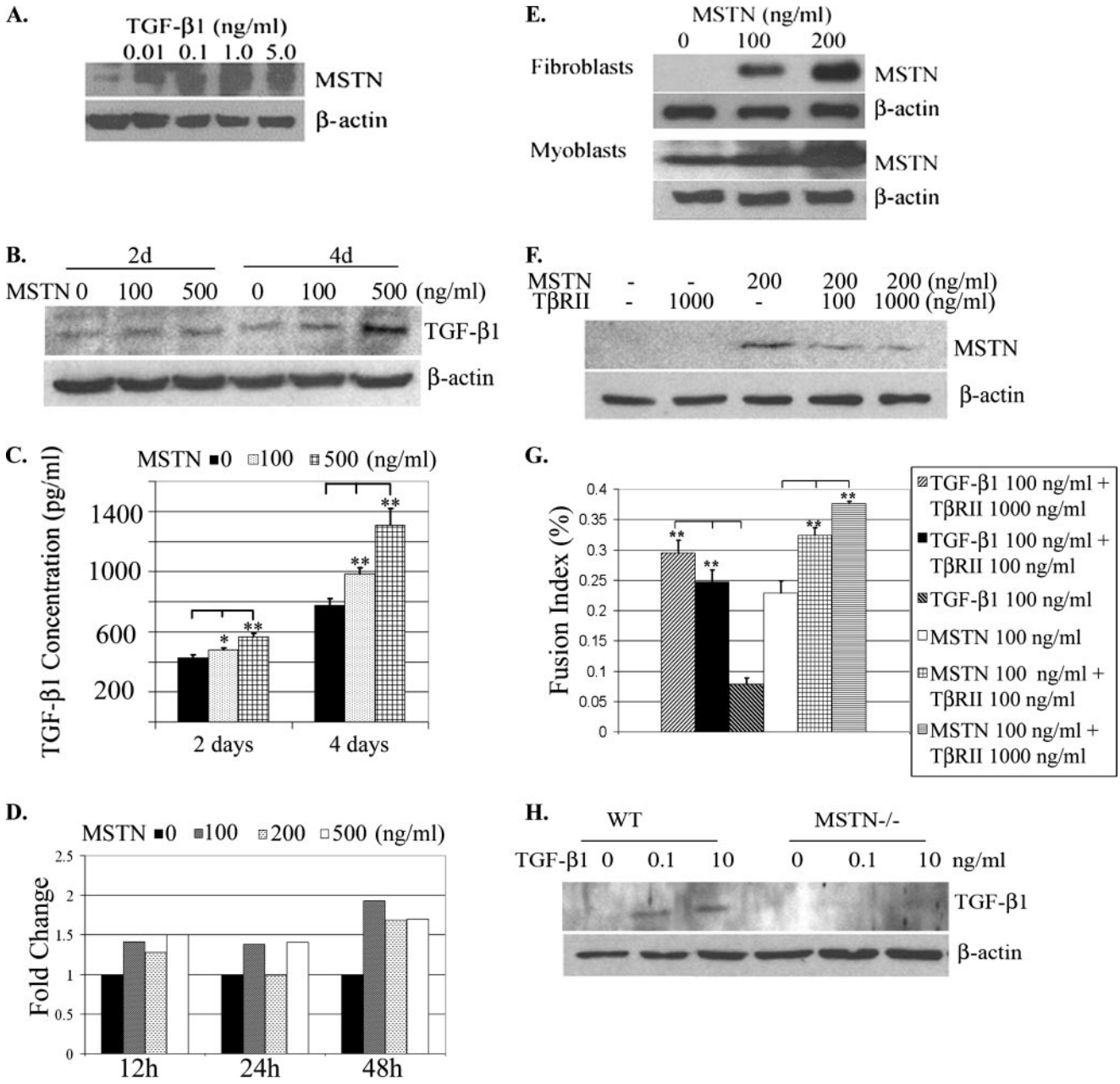

FIGURE 4. **The relationship between TGF-β1 and MSTN *in vitro*.** *A*, Western blot analysis of MSTN expression in C2C12 myoblasts treated with different concentrations of TGF-β1 ranging from 0 to 5.0 ng/ml for 48 h. *B*, C2C12 myoblasts were treated with different concentrations of MSTN in DM. Cell lysates were collected at 2 and 4 days to examine TGF-β1 expression by Western blot; *C*, while the conditioned medium was collected at the same time points, the levels of TGF-β1 in the medium were also analyzed by ELISA. *D*, Q-RT-PCR for TGF-β1 after MSTN treatment (100, 200, and 500 ng/ml) in PP1 fibroblasts. *E*, the level of MSTN expression in PP1 fibroblasts *and C2C12 myoblasts* treated with MSTN recombinant protein. *F*, Western blots were used to determine MSTN expression level in PP1 fibroblasts after cells were treated with either MSTN or both MSTN and soluble TβRII for 48 h. *G*, C2C12 myoblasts were cultured in DM with different treatments, TGF-β1, MSTN, TGF-β1 and TβRII, or MSTN and TβRII, for 4 days. Fusion indexes were used to access impacts of treatments on C2C12 myoblast differentiation. *H*, myoblasts isolated from WT, and MSTN$^{-/-}$ GMs were grown for 48 h under stimulation by TGF-β1. Western blot analysis was used to detect TGF-β1 expression in WT and MSTN $^{-/-}$ cells (*, $p < 0.05$; **, $p < 0.01$).

TGF-β1 recombinant protein was able to stimulate autocrine expression of TGF-β1 in MSTN$^{-/-}$ muscle cells as it does in C2C12 myoblasts (20). We observed that exogenous TGF-β1 could induce its autocrine expression in WT primary myoblasts but not on primary MSTN$^{-/-}$ myoblasts (Fig. 4*H*).

In vivo, We observed co-expression of TGF-β1 (*green*) and MSTN (*red*) in degenerative myofibers 1 and 3 days after laceration injury (*white arrow*, Fig. 5*A*). By day 5, MSTN was detected mainly in the nuclei of the regenerating myofibers (*white arrowhead*) with the exception of a few MSTN-positive necrotic myofibers, whereas TGF-β1 was present in the surrounding ECM (*white arrow*). MSTN was still detected in the nuclei of regenerating myofibers 21 days after injury (*white arrow*, Fig. 5*A*). The injection of MSTN into non-injured GMs induced TGF-β1 expression in the myofibers at 4, 10, and 24 h after injection. As shown in Fig. 5*B*, MSTN (*red*) and TGF-β1 (*green*) were co-expressed in myofibers at

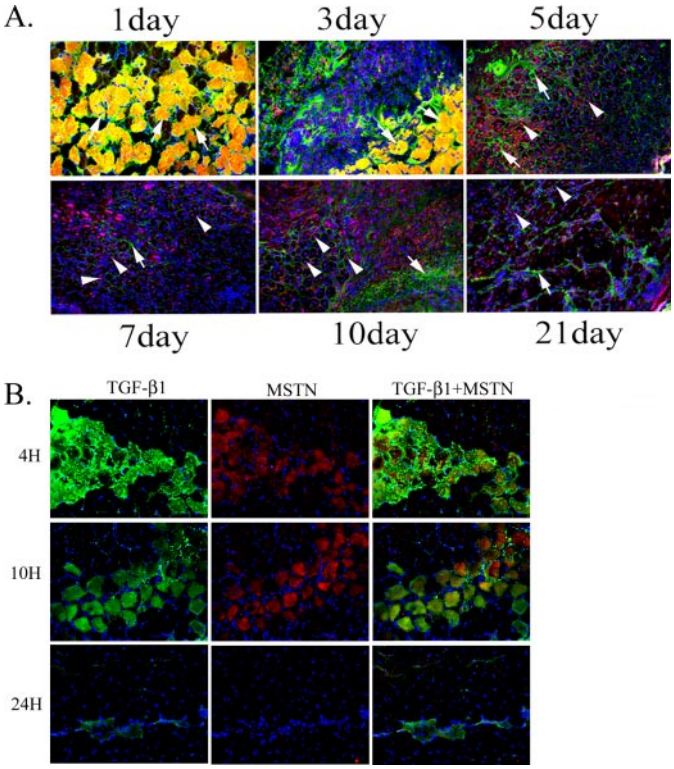

FIGURE 5. **The relationship between TGF-β1 and MSTN *in vivo*.** *A*, both GMs of each adult WT mouse underwent laceration injury. Mice GMs were harvested at the indicated times. Double staining of TGF-β1 (*green*) and MSTN (*red*) was performed. In the 1- and 3-day images, a *white arrow* indicates degenerative myofibers. At all other time points, the *white arrow* and *arrowhead* indicate ECM and nuclei of myofibers, respectively. *B*, co-localization of TGF-β1 and MSTN in myofibers after recombinant MSTN protein injection. We injected 1000 ng of MSTN protein in 10 μl of PBS into GMs of WT mice. Mice were sacrificed at different time points after injection. Frozen sections of GMs were double-stained with anti-TGF-β1 and anti-MSTN antibodies (magnification, ×200).

4 and 10 h. After 24 h, MSTN disappeared, and only a few TGF-β1-positive myofibers could be observed.

DCN Counteracts the Effect of MSTN—As previously shown in Fig. 1*A*, 0.1 μg/ml MSTN significantly stimulated PP1 fibroblast proliferation. This dosage was selected to examine whether DCN could reduce the proliferative influence of MSTN on PP1 fibroblasts. After PP1 fibroblasts were incubated with MSTN and exposed to varying concentrations of DCN for 48 h, MTT assay revealed that the addition of DCN significantly repressed the stimulatory effect of MSTN on PP1 proliferation in a dose-dependent manner as expected (Fig. 6*A*). These findings are comparable to a previous report showing that DCN blocked the stimulatory effect of TGF-β1 on PP1 fibroblasts (27).

Our earlier results indicated that MSTN induced its own expression, in an autocrine manner, in PP1 fibroblasts (Fig. 4*E*). Therefore, we examined the ability of DCN to block the MSTN autocrine expression in PP1 fibroblasts. As previously shown, PP1 fibroblasts that were not treated with MSTN failed to express detectable MSTN protein, whereas PP1 fibroblasts treated with MSTN showed a high level of MSTN expression in comparison to the control (Figs. 4*E* and 6*B*). However, DCN decreased MSTN autocrine expression by PP1 fibroblasts in a dose-dependent manner (Fig. 6*B*).

Our previous experiments showed that 1 μg/ml MSTN almost completely inhibited myoblast differentiation (data not shown). Therefore, we chose this dose to assess whether DCN treatment could reverse MSTN-inhibited myogenic differentiation in C2C12 cells. Except for the control cells, the cultures were treated with DCN alone or 1 μg/ml MSTN combined with increasing concentrations of DCN (0–50 μg/ml). Following a 5-day incubation, DCN-treated groups (data not shown) and controls showed widespread myosin heavy chain-positive myotubes, whereas cells treated with MSTN alone contained only a few myotubes (Fig. 6*C*). The addition of DCN reversed the inhibition of MSTN on myogenic differentiation, as indicated by the increase in the number and size of myotubes in comparison to the MSTN-treated group (Fig. 6*C*). Measurements showed that DCN treatment promoted C2C12 myoblast differentiation by significantly increasing fusion indexes in a dose-dependent manner (Fig. 6*D*), suggesting that DCN attenuated the inhibitory effect of MSTN and, thereby, stimulated myoblast fusion.

Inhibitory Effects of DCN on MSN May Be Mediated by FLST—To further explore whether DCN regulated MSTN activity via an intermediate molecule, we investigated the effect of DCN on the expression of FLST, which is able to bind to MSTN and suppress its activity (46). We found an up-regulation of FLST expression by C2C12 myoblasts 48 and 72 h after addition of 10 μg/ml DCN (Fig. 7*A*). Our results also revealed the ability of FLST to stimulate myogenic differentiation, which was demonstrated by the presence of larger myotubes containing more nuclei in comparison to the control group (Fig. 7*B*). In a dose-dependent manner, FLST treatment led to a significant increase in fusion index (Fig. 7*C*) compared with the control group, suggesting that FLST promotes myogenic differentiation and accelerates the maturation of myotubes.

DISCUSSION

MSTN has been drawing more and more attention due to mounting evidence indicating that inhibition of MSTN significantly improves skeletal muscle diseases such as muscle dystrophy. But, the role of MSTN in injured skeletal muscle and its relationships with other molecules such as TGF-β1 and DCN (important key factors in muscle healing) remain unknown. Recent studies reported by Yamanouchi *et al.* (47) highlight the expression of MSTN in fibroblasts in injured skeletal muscle, suggesting that fibroblasts may be a source of MSTN. Previously, we have shown that TGF-β1 significantly promotes proliferation of PP1 fibroblasts (27). Here, our *in vitro* study shows that MSTN activates fibroblasts by stimulating fibroblast proliferation and inducing their expression of α-SMA analogous to that of TGF-β1. Like TGF-β1 (48), MSTN may transiently attract fibroblasts into an injury site, further inducing them to express MSTN in an autocrine fashion; they then differentiate into myofibroblasts, thereby accelerating the deposition of the ECM. Researchers widely believe that prolonged presence and excessive activity of myofibroblasts is associated with the abnormal accumulation of ECM components in injured and diseased tissue (49, 50). Moreover, MSTN has been shown to induce procollagen (types Iα1, Iα2, and IIIα1), mRNA, and FN protein expression in PP1 fibroblasts. McCroskery *et al.* (51) recently confirmed the correlation of MSTN expression to the

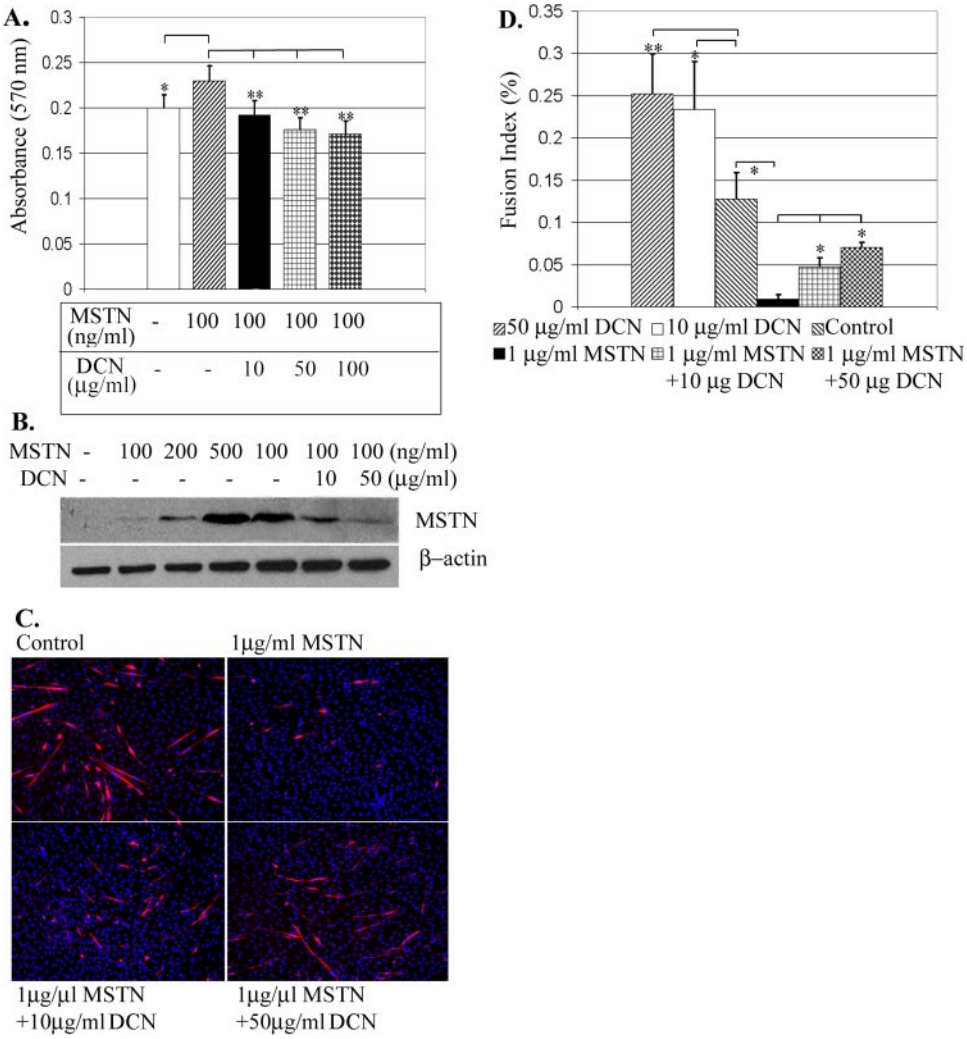

FIGURE 6. **DCN blocks the effects of MSTN on PP1 fibroblasts and C2C12 myoblasts.** *A*, PP1 fibroblasts were treated for 48 h with 100 ng/ml MSTN or combinations of MSTN and DCN. Non-treated cell cultures were used as a control. MTT assay was performed to assess cell proliferation. *B*, after incubation of PP1 fibroblasts with MSTN, or a combination of MSTN and DCN, Western blot analysis was performed to determine whether DCN reduced the autocrine expression of MSTN in PP1 fibroblasts stimulated with MSTN. *C*, C2C12 myoblasts were cultured without treatment, with 1 μg/ml MSTN alone, or co-incubated with 1 μg/ml MSTN and different concentrations of DCN for 5 days. Myotubes were monitored by anti-skeletal myosin heavy chain immunostaining; nuclei were stained by Hoechst 33258 (magnification, ×100). *D*, fusion indexes were determined to estimate the differentiation capacity of C2C12 myoblasts in response to different treatments.

formation of fibrosis by showing less fibrosis formation in the notexin-damaged tibialis anterior muscle in MSTN$^{-/-}$ mice 4 weeks after injury as compared with WT mice. Given the results collected in our *in vitro* study, we hypothesized that a lack of MSTN in knock-out mice would decrease the proliferation of fibroblasts and reduce their production of collagenous tissue in injured skeletal muscle. This was made evident by a significant decrease in the formation of fibrosis in MSTN$^{-/-}$ mice at 2 and 4 weeks after injury when compared with WT mice. Moreover, we found an elevated expression level of DCN, an inhibitor of TGF-β1, in injured MSTN$^{-/-}$ skeletal muscles compared with injured WT muscles at 2 weeks after injury. In accordance with this result, increased DCN mRNA has been observed in regenerating MSTN$^{-/-}$ muscle (51). Increased DCN might inhibit the effect of TGF-β1, thereby partially explaining the reduced fibrosis and enhanced regeneration in injured MSTN$^{-/-}$ muscle. To understand the mechanism by which MSTN$^{-/-}$ muscle displays less fibrosis than WT muscle after injury, the expression levels of TGF-β1 in injured WT mice *versus* that expressed in injured MSTN$^{-/-}$ mice should be compared more closely.

As members of the TGF-β superfamily, TGF-β1 and MSTN share many similarities in structure, signaling pathway, and function (52, 53). It has also been shown that TGF-β1 plays a critical role in skeletal muscle fibrosis after injury (20, 26–32). Because both TGF-β1 and MSTN promote fibrosis, it is very important to understand the potential relationships between these two molecules. Recent reports demonstrated that exogenous TGF-β1 strongly stimulated the expression of MSTN in C2C12 myoblasts (44). In fact, our *in vitro* data show that TGF-β1 increases MSTN expression in C2C12 myoblasts (and *vice versa*), and TGF-β1 and MSTN are found to co-localize in the same myofibers shortly after MSTN injection or after injury.

We found that MSTN is able to induce its autocrine expression in both fibroblasts and myoblasts. In the presence of soluble TβRII, MSTN autocrine expression in fibroblasts is decreased. We have known that MSTN inhibits C2C12 myoblast differentiation. When TβRIIs are blocked by soluble TβRII, the ability of MSTN to inhibit C2C12 myoblast differentiation is reduced. Apart from that, Q-RT-PCR results show that MSTN also stimulates TGF-β1 mRNA expression in PP1 fibroblasts. Our previous study has shown that TGF-β1 is able to induce autocrine expression of TGF-β1 in C2C12 myoblasts (20), nevertheless, our present data revealed that TGF-β1 failed to induce its autocrine expression in MSTN$^{-/-}$ primary muscle cells. Although TGF-β1 and MSTN may target different cell membrane receptors (52), our results suggest that they may also bind to the same receptor, indicating that their signaling may be somehow related. It is likely, then, that the inducement of skeletal muscle fibrosis by TGF-β1 is partially mediated by its interaction with MSTN. However, the mechanism by which TGF-β1 interacts with MSTN to cause fibrosis warrants further investigation.

Satellite cells serve as a reservoir of myogenic progenitor cells for the repair and maintenance of skeletal muscle. MSTN negatively regulates self-renewal and differentiation of satellite

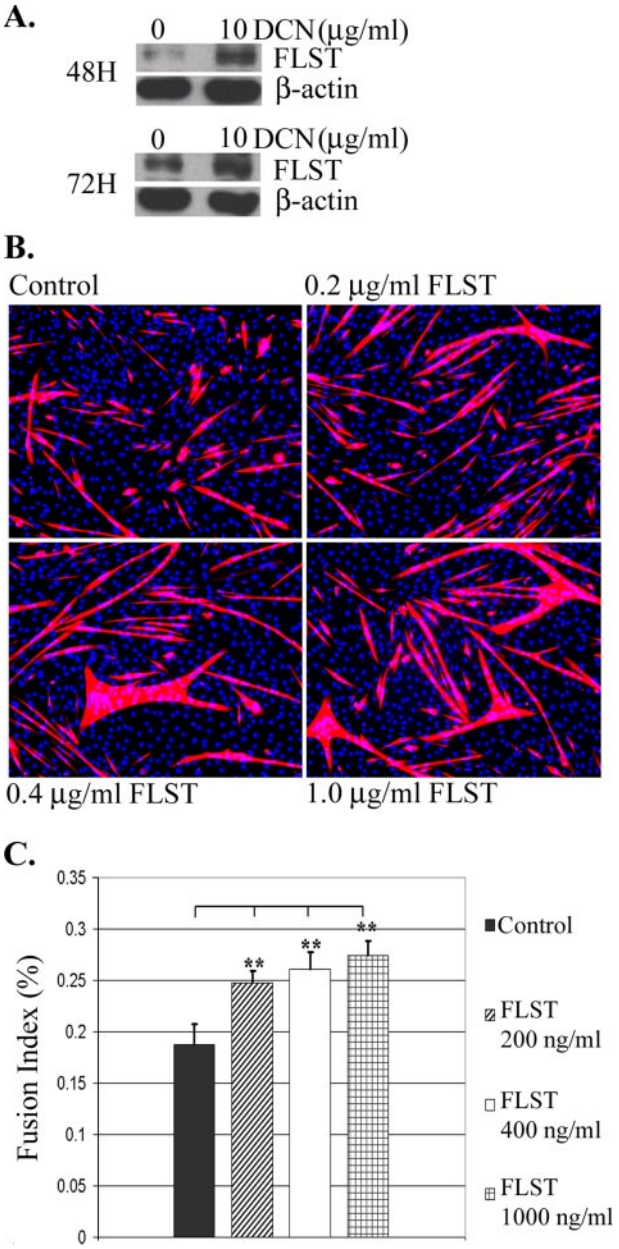

FIGURE 7. **The elevated FLST expression by DCN and the capacity of FLST to enhance myogenic differentiation of C2C12 myoblasts.** *A*, DCN increased the expression of FLST in C2C12 myoblasts 48 and 72 h after treatment. *B*, immunofluorescence analysis of myotubes. C2C12 myoblasts were maintained in DM for 5 days in the presence of different concentrations of FLST. Myotubes were double-labeled with an antibody recognizing skeletal myosin heavy chain and with the fluorescent nuclear dye Hoechst 33258 (magnification, ×100). *C*, fusion indexes were calculated to evaluate the degree of C2C12 myoblast differentiation upon FLST stimulation.

cells (54) and decreases the expression of members of the basic helix-loop-helix muscle regulatory factors (MRF) (MyoD, Myf5, mrf4, and myogenin) (43, 55). MSTN$^{-/-}$ mice show an increased number of satellite cells activated and differentiated toward a myogenic lineage (54). In this study, our data demonstrate that MSTN$^{-/-}$ mice contain regenerating myofibers with significantly larger diameters than WT mice at 2 and 4 weeks after GM laceration. The increased number of satellite cells in MSTN$^{-/-}$ mice could, in part, explain the enhanced regeneration revealed by the larger diameter of regenerated myofibers in MSTN$^{-/-}$ mice compared with WT mice. Indeed, it has been reported that blocking MSTN signals by isolating myoblasts from transgenic mice carrying the mutated MSTN receptor results in improved success of myoblast transplantation in *mdx* mice compared with normal myoblasts (56). Our results show that MSTN$^{-/-}$ LTP more readily undergo myogenic differentiation *in vitro* and regenerate skeletal muscle *in vivo* in a more effective manner than wild-type cells.

Furthermore, high levels of MSTN protein have been reported within necrotic fibers in the skeletal muscles of rats damaged by notexin (57), and Western blot analysis revealed the up-regulation of MSTN protein at early time points following notexin-induced injury in rat skeletal muscle (58). Interestingly, it has been shown that MSTN interferes with the chemotaxis of macrophages *in vitro* (51); recombinant MSTN protein significantly reduces the migration of macrophages and myoblasts toward chemoattractants *in vitro*, which likely promotes skeletal muscle regeneration (51). These results suggest that MSTN could impede recruitment of macrophages and myoblasts into the injured site *in vivo*. Macrophages infiltrate damaged tissue to remove debris that could hinder muscle regeneration. Macrophages also secrete a variety of growth factors and cytokines that have chemotactic and/or mitogenic effects on muscle precursor cells, thereby accelerating muscle regeneration (59–63). Compared with WT mice, MSTN$^{-/-}$ mice have shown elevated recruitment of macrophages and myoblasts and an accelerated inflammatory response after muscle injury (51). These results suggest that the earlier initiation of skeletal muscle regeneration in the injured skeletal muscle of MSTN$^{-/-}$ mice compared with the injured muscle of WT mice may be due, in part, to accelerated removal of muscle debris. When we monitored the expression of MSTN at the injured site for up to 30 days after injury, we observed an intense expression of MSTN in the cytoplasm of degenerative myofibers 1 and 3 days after laceration. On day 5 after injury, MSTN signal was detected in the cytoplasm of regenerating myofibers. Our results show that the MSTN signal decreases with maturation of regenerating myofibers. Interestingly, there is strong MSTN immunostaining in regenerating small myotubes lacking basal lamina 7 and 14 days post-injury. During skeletal muscle healing (following active muscle regeneration at early time points after injury) fibrosis initiates ~1 week post-injury, and peaks at 4 weeks (10, 15, 64). Li *et al.* (20) reported that some regenerating myofibers probably differentiate into myofibroblasts to contribute the formation of fibrosis. This correlation between fibrosis development and increased MSTN and TGF-β1 expression (20) in the early phase of healing may suggest the differentiation of regenerating myotubes/myofibers into myofibroblasts and a potential interaction between TGF-β1, MSTN, and DCN, as previously hypothesized (65).

MSTN-positive signals were also seen within the nuclei of the newly formed fibers at 5, 7, and 10 days post-injury. The nuclear localization of MSTN is supported by previous studies indicating that MSTN was detected in the nuclei of myoblasts and myotubes (66). Consequently, MSTN protein might modulate the muscle fiber regeneration process through the early events of phagocytosis and inflammation (57) and later control myofiber maturation. In this way, MSTN seems to act as a reg-

ulatory molecule that is produced by the tissue to specifically suppress and control the size of muscle growth and development (67).

DCN, a small chondroitin-dermatan sulfate leucine-rich proteoglycan, exists ubiquitously in the ECM. Due to its binding to and inhibition of TGF-β1, DCN has been used as a potent anti-fibrosis agent in various organs and tissues (26, 27, 37–40), including skeletal muscle (26, 27). However, the ability of DCN to regulate MSTN activity is still unknown. DCN, which is composed of a core protein and a single glycosaminoglycan chain (68, 69), has the ability to bind to TGF-β1 due to the fact that the core protein of DCN contains two binding sites for TGF-β1 (70). Similarly, Miura *et al.* (65) have shown that DCN, or the core protein of DCN, directly binds to active MSTN molecules to block MSTN-mediated inhibition of C2C12 myoblast proliferation. The actual location of the MSTN binding site in the DCN core protein and evidence that shows whether TGF-β1 and MSTN competitively bind to DCN are topics for further investigation. Of further interest is the possibility that DCN may regulate MSTN by influencing another intermediate molecule like FLST, an antagonist of MSTN (46). Our results not only show that DCN reduces the effects of MSTN on fibroblasts and myoblasts, but also indicates that it stimulates the expression of FLST in C2C12 myoblasts. Exogenous FLST then stimulates C2C12 myoblast differentiation, which is probably due to FLST's neutralization of endogenous MSTN. These results indicate that the effect of DCN on MSTN may be related to the up-regulation of FLST, which would consequently suppress MSTN activity. Nevertheless, more experiments that would, for example, examine the effect of DCN on FLST knock-out cells, need to be done to establish the role of FLST in DCN-inhibited MSTN activity. Furthermore, we have shown that TGF-β1 probably plays a role in the MSTN signaling pathway, because TGF-β1-soluble receptor antagonizes, at least in part, the effect of myostatin on muscle cells. Overall, DCN probably regulates MSTN activity via three ways: (i) directly binding MSTN, (ii) indirectly down-regulate MSTN by binding to TGF-β1, and (iii) indirectly down-regulating MSTN by stimulating FLST expression.

In summary, our results suggest the following: (i) MSTN stimulates the formation of fibrosis in skeletal muscle after injury, (ii) TGF-β1 and MSTN up-regulate the expression level of each other, and (iii) DCN is capable of inhibiting MSTN activity as it does for TGF-β1. These results, combined with the fact that TGF-β1 plays a key role in skeletal muscle fibrosis and that DCN reduces fibrosis in injured skeletal muscle, suggest that TGF-β1 and MSTN probably act together; they synergistically amplify the fibrotic process in injured or diseased skeletal muscles resulting in greater fibrosis than either could induce individually.

Our findings may help to further increase the understanding of the mechanism by which $MSTN^{-/-}$ mice show decreased fibrosis and enhanced regeneration after injury and suggest that the inhibition of MSTN might be a new therapeutic approach for improving skeletal muscle healing through enhancement of regeneration and reduction of fibrosis.

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DECORIN INTERACTS WITH MYOSTATIN ACTIVITY – IMPLICATIONS FOR SKELETAL MUSCLE HEALING

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Introduction:

Skeletal muscle injuries are one of the most common injuries occuring in field of sports medicine. Muscle injuries can heal spontaneously via regeneration, but fibrosis usually impedes this process which results in incomplete functional recovery. We have demonstrated that TGF-β1 plays a major role in the initiation of fibrosis in injured muscle [1]. Decorin, a small chondroitin-dermatan sulphate leucine-rich proteoglycan, has been shown to improve skeletal muscle healing histologically and physiologically through a concomitant increase in muscle regeneration and a decrease in fibrosis [2]. It is widely accepted that the antifibrotic effect of decorin primarily results from its capability of directly binding to and neutralizing TGF-β1 [3]. The identification of a new TGF-β family member, myostatin (MSTN) [4], has inspired us to further explore the mechanism by which decorin improves skeletal muscle healing. Our unpublished data showed that 1) MSTN stimulates proliferation and myofibrotic differentiation of fibroblasts in vitro; 2) that the injured skeletal muscle of MSTN knockout (MSTN-/-) mice contains significantly less fibrous scar tissue than observed in the injured muscle of normal wild-type (WT) mice and 3) regenerating myofibers of MSTN-/- mice are significantly larger in diameter than those in the injured muscle of WT mice. Thus MSTN, like TGF-β1, appears to play an important role in fibrosis. We performed this study to investigate whether decorin could inhibit the activity of MSTN as it does with TGF-β1.

Methods:

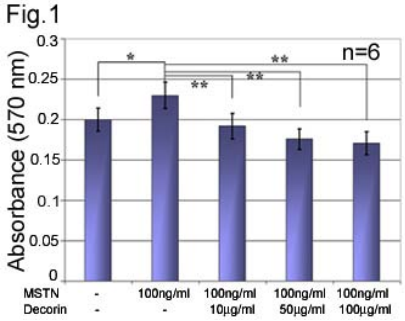
Proliferation assay and Western blot analysis: PP1 fibroblasts were plated onto collagen-coated 96-well plates for cell proliferation analysis. Following overnight attachment, normal medium was replaced with serum free medium plus serum replacement (Sigma, St Louis, MO) in the presence of varying concentrations of decorin and 100ng/ml of recombinant human MSTN. After an additional incubation for 48 h, an MTT cell proliferation assay kit (Roche Diagnostics, Germany) was used to measure cell proliferation (n = 6) following instructions from the manufacturer. Western blot was used to examine β-actin and MSTN expression. To test whether decorin neutralized the inhibitory effect of MSTN on myogenic cell differentiation, C2C12 myoblasts were seeded in 12-well plates at a density of 10,000cells/well. After overnight incubation, medium was replaced with fresh differentiation medium (DM) containing DMEM, 2% HS, and 1% P/S, with or without addition of 1μg/ml MSTN. This was concomitant with the addition of 0-100 μg/ml decorin (n = 3). Cells were cultured for another 5 days. DM, MSTN, and decorin were changed every other day.

Injury model: All experiments in this study were in accordance with research protocols approved by the ARCC of Children's Hospital of Pittsburgh. MSTN-deficient mice (MSTN-/-) and C57BL/6J wild-type mice (control) were used to establish an injury model of skeletal muscle laceration [2]. GMs of both MSTN-/- and wild-type mice were harvested 2 weeks after laceration. Decorin immunostaining was performed to evaluate decorin expression in injured skeletal muscle. Student's *t*-test or one way ANOVA were used to determine significance ($P < 0.05$).

Results:

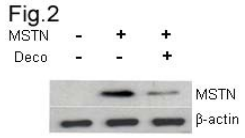
Blockade of MSTN effect on fibroblasts by Decorin:. After PP1 fibroblasts were incubated with MSTN and varying concentrations of decorin for 48 h, MTT assay revealed that 0.1μg/ml MSTN significantly stimulated PP1 fibroblast proliferation whereas the addition of decorin significantly repressed the MSTN stimulatory effect on PP1 proliferation (Fig 1).

Fig.1



Decorin blocks MSTN autocrine expression in fibroblasts: After 48 h incubation, PP1 fibroblasts did not express detectable MSTN protein, and PP1 fibroblasts treated with MSTN showed a high level of MSTN expression. However, MSTN expression in PP1 fibroblasts treated with both MSTN and decorin was reduced to only a detectable level (Fig 2).

Fig.2



Blockade of MSTN effect on C2C12 myoblast differentiation by Decorin

Following a 5-day incubation, controls showed widespread skeletal muscle heavy chain (MHC) positive myotubes whereas cells treated with MSTN alone contained few myotubes (Fig 3). The addition of decorin reversed the inhibition of MSTN on myogenic differentiation (Fig 3). Quantification showed that decorin treatment significantly promoted C2C12 myoblast fusion and increased the number and size of the myotubes in the presence of MSTN (Fig 3) in a dose dependent manner

Elevated decorin expression in injured MSTN-/- mice

Immnunohistochemical staining revealed that there was a significantly higher level of decorin expression in the regenerating skeletal muscle of MSTN-/- mice than that of WT mice 2 weeks after injury (Fig 4).

Fig.3

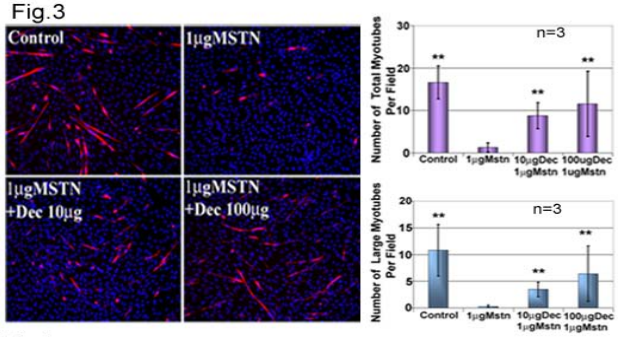
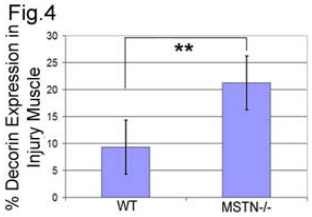


Fig.4



Discussion:

In this study, we showed that decorin blocked the effects of MSTN on both fibroblasts and myoblasts in vitro. Decorin reduced the stimulating effects of MSTN on fibroblasts and the MSTN autocrine expression of fibroblasts. Moreover, decorin rescued the inhibitory effect of MSTN on myoblast differentiation. The ability of decorin to bind to TGF-β1 is due to the fact that core protein of decorin contains two binding sites for TGF-β1. Similarly, it has been recently shown that decorin, or the core protein of decorin, can directly bond to active MSTN molecules to block MSTN-mediated inhibition of C2C12 myoblast proliferation [5]. The location of the MSTN binding site in the decorin core protein and whether TGF-β1 and MSTN competitively bind to decorin are topics for further investigation. These findings provide us a better understanding of how decorin can improve skeletal muscle healing after injury.

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FOLLISTATIN IMPROVES SKELETAL MUSCLE HEALING AFTER INJURY

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INTRODUCTION

Skeletal muscle injuries account for 10 to 30% of all sports related injuries. Muscle injuries can heal via spontaneous regeneration; however, as a result of severe injury, incomplete functional recovery can increase the time an athlete is off the field. Decorin has been shown to effectively improve skeletal muscle healing by reducing fibrosis and accelerating skeletal muscle regeneration [1]. Aside from decorin, Follistatin (FLST) has been drawing more attention do to the fact that increasing knowledge about this molecule reveals it as a promising new target for therapeutic improvement of skeletal muscle healing. FLST was initially described as an inhibitor of follicle-stimulating hormone decades ago. More and more evidence revealed that FLST is capable of binding and neutralizing many members of the TGF-β superfamily such as myostatin (MSTN) and activin [2, 3]. Recently, it was found that FLST overexpression mice (FLST OE) showed dramatic increase in skeletal muscle mass compared to wild-type control mice [4]. Activin is implicated in the formation of fibrosis in many tissues and organs such as skin, liver, and kidney [2, 3]. Furthermore, our unpublished data demonstrated that MSTN is a fibrosis stimulator in the skeletal muscle and blocking MSTN significantly reduces the formation of fibrosis in injured skeletal muscle of mice. Taken together, we hypothesize that FLST may improve the healing of injured skeletal muscle. In this study, transgenic FLST OE mice were used to investigate the influence of FLST on skeletal muscle healing.

METHODS

Myoblast differentiation assay: C2C12 myoblasts were plated onto collagen-coated 12-well plates with normal medium overnight. The following day, the medium was replaced with low serum medium (2% horse serum) plus different concentrations of FLST (Sigma, St. Louis, MO). The cells were cultured for 6 additional days. Medium and recombinant FLST protein were changed every other day. Myotubes were monitored by myosin heavy chain (MHC) immunostaining, and the fusion capacity of C2C12 myoblasts was evaluated by determining the number of myonuclei per myotube (n = 3).

Western blot: C2C12 myoblasts were plated onto collagen-coated 6-well plates. Following overnight attachment, normal medium was replaced with low serum medium in the presence of decorin (Sigma, St. Louis, MO). Protein samples were collected at 48 and 72h and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Goat anti-follistatin antibody (Santa Cruz Biotechnology, INC. Santa Cruz, CA) was used to detect FLST expression in C2C12 myoblasts

Animal model: All experiments in this study were approved by the Children's Hospital of Pittsburgh IACUC. C57BL/6J wide-type (WT) and FLST OE mice (7 to 8 weeks of age) were used for all experiments (n = 6). Both gastrocnemius muscles (GMs) of each mouse underwent bilateral laceration [1]. The GMs were harvested 4 weeks after laceration, and Masson's Trichrome staining (nuclei [black], muscle [red], collagen [blue]) was performed to identify fibrous scar tissue in the injured muscles. Northern Eclipse software (Empix Imaging, Inc.) was used to measure areas of fibrous scar tissue and regenerated muscle within the injury site.

Statistics: One-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison test or Student's *t* test was used to determine significance ($P < 0.05$) throughout this study. (*) represents that $P < 0.05$, and (**) represents that $P < 0.01$;

RESULTS

FLST induces large myotube formation in vitro: Our results reveal the ability of FLST to stimulate myogenic differentiation as shown in Fig 1. Myotubes treated with FLST were larger and contained more nuclei than the control (Fig. 1A). FLST treatment leads to a significant increase in the number of nuclei per myotubes in a dose dependent manner (Fig. 1B) compared to control, suggesting that FLST promotes myogenic fusion and accelerates the maturation of myotubes.

Reduced fibrosis in FLST OE mice after GM laceration: The results of Masson's Trichrome histochemistry revealed significantly less fibrous scar tissue in the FLST OE GMs than in the WT GMs (Fig. 2A, B).

Decorin stimulates FLST expression in C2C12 myoblasts: We found that decorin up-regulated FLST expression in C2C12 myoblasts after 48h and 72h of culture (Fig.3).

Fig.1A

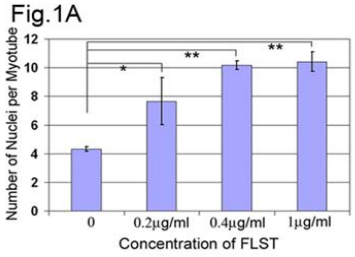


Fig.1B.

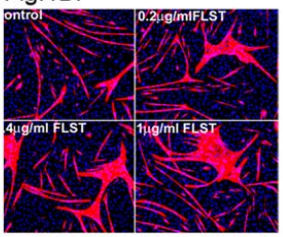


Fig.2A

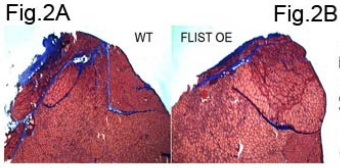


Fig.2B

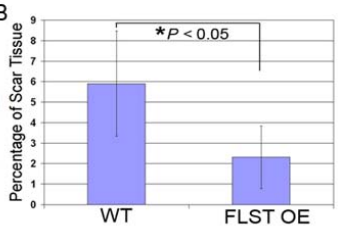
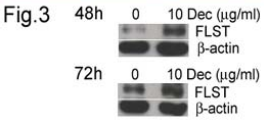


Fig.3



DISCUSSION

We have demonstrated that TGF-β1 plays a significant role in both the initiation of fibrosis and the induction of myofibroblastic differentiation by myogenic cells in injured skeletal muscle [6]. Although, FLST improves skeletal muscle healing, it does not seem to block fibrosis in injured skeletal muscle through inhibition of TGF-β1. Our result showed that FLST failed to block the inhibition that TGF- β1 has on myogenic differentiation (data not shown). Most likely, FLST inhibits the formation of fibrosis by antagonizing the activities of activin and MSTN [2, 3]. FLST overexpression improves skeletal muscle healing by reducing fibrosis. Although FLST significantly increases myoblast differentiation in vitro, FLST OE mice did not show increased diameter of regenerating myofibers compared to WT mice at 4 weeks after GM laceration. This suggests that FLST is involved with an alternative mechanism for skeletal muscle healing. Moreover, we also showed that decorin treatment elevated FLST expression in C2C12 myoblasts. It seems likely that, apart from neutralizing TGF- β1, decorin also reduces fibrosis in injured skeletal muscle by up-regulating the intermediate molecule, FLST. The skeletal muscle healing process is a complex process, in which many molecules interact with each other, and our findings provide insight into one aspect of this network.

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Skeletal Muscle Fiber Type Conversion during the Repair of Mouse Soleus: Potential Implications for Muscle Healing after Injury

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ABSTRACT: We used a mouse model of cardiotoxin injury to examine fiber type conversion during muscle repair. We evaluated the soleus muscles of 37 wild-type mice at 2, 4, 8, and 12 weeks after injury. We also used antibodies (fMHC and sMHC) against fast and slow myosin heavy chain to classify the myofibers into three categories: fast-, slow-, and mixed (hybrid)-type myofibers (myofibers expressing both fMHC and sMHC). Our results revealed an increase in the percentage of slow-type myofibers and a decrease in the percentage of fast-type myofibers during the repair process. The percentage of hybrid-type myofibers increased 2 weeks after injury, then gradually decreased over the following 6 weeks. Similarly, our analysis of centronucleated myofibers showed an increase in the percentage of slow-type myofibers and decreases in the percentages of fast- and hybrid-type myofibers. We also investigated the relationship between myofiber type conversion and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α). The expression of both PGC-1α protein, which is expressed in both the nucleus and the cytoplasm of regenerating myofibers, and sMHC protein increased with time after cardiotoxin injection, but we observed no significant differential expression of fMHC protein in regenerating muscle fibers during muscle repair. PGC-1α-positive myofibers underwent fast to slow myofiber type conversion during the repair process. These results suggest that PGC-1α contributes to myofiber type conversion after muscle injury and that this phenomenon could influence the recovery of the injured muscle. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 25:1534–1540, 2007

Keywords: PGC-1α; fiber type conversion; muscle injury and repair

INTRODUCTION

Mammalian skeletal muscles contain myofibers that differ in terms of function, mitochondrial content, and metabolic properties.[1] Slow-twitch myofibers display long contraction times, contain a large number of mitochondria, and rely on the oxidative phosphorylation metabolic pathway as a main energy source.[2] In contrast, fast-twitch myofibers display short contraction times, contain a small number of mitochondria, and generate ATP primarily through glycolysis.[2]

Skeletal muscle exhibits tremendous plasticity that allows myofiber type conversion in response to a chronic change in contractile demands.[3–6] For example, repeated mechanical overload and endurance training increases the percentage of slow myofibers. Researchers can mimic this shift in the percentages of slow and fast myofibers by using electrical stimulation of motor nerves or cross-reinnervation of muscles with nerves supplying slow-twitch fibers. Salvani et al. analyzed the regenerated gastrocnemius muscles of mice 8 months after myotoxin injury.[7] Myotoxin injury induced muscle fiber type conversion from fast- to slow-type myofibers, and the injured muscles contained a higher percentage of small fast- and slow-type myofibers than did saline-treated muscles.[7] However, Salvani's group did not examine the underlying mechanism of fiber type conversion.[7]

Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is expressed in several tissues, including skeletal muscle and brown adipose tissue, and has been implicated in the development of slow myofibers in mice.[8] Russell et al. investigated the muscles of humans participating in a 6-week endurance training program

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and observed an increase in the PGC-1α protein content in the participants' skeletal muscle. This increase was accompanied by a shift in fiber composition: the percentage of slow fibers increased while the percentage of fast fibers decreased.[9]

Together, these findings led us to hypothesize that muscle injury induces an increase in PGC-1α expression, which is implicated, at least in part, in the conversion of fast-type myofibers into slow ones. We examined the mouse soleus muscle for this study because it normally contains similar numbers of slow and fast fibers. The aim of this study was to evaluate the fiber type composition of the soleus muscles after an induced cardiotoxin injury in a 5-week-old mouse that undergoes repair over 12 weeks and investigate the relationship between the myofiber type transition and the PGC-1α expression in the soleus during the 12-week repair period. Next, we examined the potential relationship between myofiber type conversion and PGC-1α expression in injured muscle undergoing repair.

METHODS

Animal Model

Thirty-seven mice (C57BL/6J, female, 5 weeks of age; The Jackson Laboratory, Bar Harbor, ME) were used for the soleus muscle cardiotoxin injury model. The experimental protocols were all approved by The Animal Research and Care Committee (ARCC) at Children's Hospital of Pittsburgh (ARCC Protocol #15/03). The mice were anesthetized by intraperitoneal injection of 0.03 ml Ketamine (100 mg/mL; Abbott Laboratories, Chicago, IL) and 0.01 mL of Xylazine (20 mg/mL; Phoenix, St. Joseph, MO). The right soleus muscles of the mice were injected with 1 μg of cardiotoxin (Accurate Chemical and Scientific Corporation, Westbury, NY) in 1 μL of normal saline. The mice were euthanized 2, 4, 8, or 12 weeks after injection. The soleus muscles were harvested, flash-frozen in 2-methyl butane pre-cooled in liquid nitrogen, and stored at −80°C pending histologic analysis.

Immunohistochemistry

Serial 10-μm-thick cryostat sections were made following standard techniques. For myofiber type staining, the sections were pretreated with blocking solution (Mouse-on Mouse [M.O.M.]; Vector, Burlingame, CA) to eliminate nonspecific antibody binding. Both NOQ7.5.4D, monoclonal mouse anti-slow myosin heavy chain (sMHC) antibody (1:200; Sigma, St. Louis, MO), and MY32, monoclonal mouse anti-fast myosin heavy chain (fMHC) antibody (M4276, 1:400; Sigma) were diluted with M.O.M. Previous studies have shown that NOQ7.5.4D (sMHC) antibody stained slow myofiber based on the ATPase staining[8] and MY32 (fMHC) antibody correlated to fast myofiber derived from the ATPase staining.[10] The fast and slow fibers then were visualized separately by treating the sections with a sheep anti-mouse IgG-Cy3 conjugate (Sigma) diluted 1:250 with M.O.M. and an FITC-conjugated affinity-purified horse anti-mouse IgG (H+L) (Vector) diluted 1:200 with M.O.M for 30 min at room temperature. The polyclonal rabbit anti-mouse PGC-1α antibody (AB3242; Chemicon, Temecula, CA) was diluted 1:300 with 0.1% Triton X-100/PBS and visualized by exposure to an Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (Molecular Probes, Eugene, OR) at a concentration of 1:100 for 30 min at room temperature. Negative control experiments (without the primary antibody) were performed concurrently with all immunohistochemical staining. The sections were counterstained with 4, 6-diamidino-2-phenylindole staining (DAPI, Sigma) which stains the nuclei in the sections. Fluorescent microscopy was used to visualize all of the immunofluorescence results (Nikon E800), and muscle fiber composition was determined by counting the red (sMHC), green (fMHC), and yellow (colocalized-hybrid) fibers under 200× magnification. Slides from five muscles were used for each group.

Western Blot Analysis

Muscle samples were homogenized and subjected to standard sample preparation procedures. The proteins were separated by 8% SDS-polyacrylamide electrophoresis gel for PGC-1α, 10% for β-actin, and 12% for fMHC and sMHC. Then the samples were transferred to nitrocellulose membranes that were used to perform immunostaining. sMHC antibody (1:1000), fMHC antibody (1:1000), and polyclonal rabbit anti-mouse PGC-1α antibody (1:1000) were applied as primary antibodies. Mouse anti-β-actin (1:8000; Sigma) was used for protein quantification. The horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL) was diluted to 1:12000 for sMHC, fMHC, and β-actin and 1:5000 for PGC-1α. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used to develop the blots, and positive bands were visualized on X-ray film. Bands on X-rays were analyzed by using image analysis software (Northern Eclipse software v.6.0; Empix Imaging, Cheektowaga, NY).

Statistics

The percentage of the different types of myofibers (total myofibers and regenerating myofibers) and the percentage of the different types of myofibers that expressed PGC-1α in the injured muscle were graphed (mean ± SD). Statistical differences in the percentages of fiber types and the fibers' protein content in the different groups were determined by two-way ANOVA. When statistical differences were observed, a Turkey multiple comparison test was used for the post hoc analysis. A P-value less than 0.05 was considered to be statistically significant.

RESULTS

Conversion of Fast into Slow Muscle Fibers in Regenerating Muscle

Noninjured soleus muscles contained three types of myofibers: fast-, slow-, and hybrid-type myofibers (Fig. 1). In the normal soleus, $53\% \pm 3\%$ of the myofibers were positive for fMHC, $35\% \pm 4\%$ were positive for sMHC, and $12\% \pm 3\%$ expressed both fMHC and sMHC. Cardiotoxin-induced injury of the soleus muscle resulted in time-dependent changes in the ratio of myofiber types (Fig. 2). The percentage of fMHC-expressing myofibers had decreased to $14\% \pm 2\%$ by 8 weeks after injury. In contrast, the percentage of sMHC-expressing myofibers had increased to $79\% \pm 1\%$ by 8 weeks after injury. The percentage of hybrid-type myofibers had increased to $33\% \pm 7\%$ 2 weeks after injury but had decreased to $6\% \pm 2\%$ by 8 weeks after injury. In contrast to observations at the earlier time points, the prevalence of the different fiber types did not change significantly between 8 and 12 weeks after injury.

Analysis of only the newly-regenerated (centronucleated) myofibers revealed a decrease in the percentages of both fMHC-expressing fibers and hybrid-type fibers. Additionally, an increase in sMHC-expressing fibers accompanied the decreasing percentages of both fMHC-expressing fibers and hybrid-type fibers (Fig. 3). Our study demonstrated that there were no changes in the regenerating muscle fibers in the ratios of fiber types between 8 weeks and 12 weeks after injury (Fig. 3).

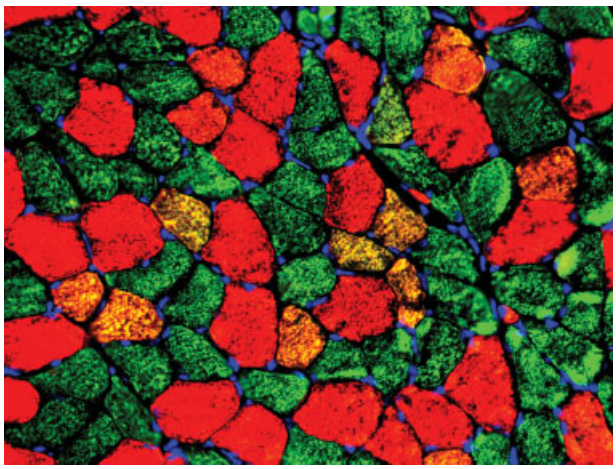

Figure 1. Immunohistochemical double staining for fast and slow myosin heavy chain (fMHC and sMHC) in normal murine soleus muscle (original magnification $\times 400$). Fast (green)-, slow (red)-, and hybrid (yellow)-type myofibers and nuclei (blue) are visible.

Increase in PGC-1α and sMHC Protein Content during Muscle Regeneration

We also investigated the expression of fMHC, sMHC, and PGC-1α protein in the soleus muscle at different times after injury by western blot (Fig. 4A). Of the total amount of β-actin present in the normal soleus, the relative expressions of fMHC, sMHC, and PGC-1α protein were $55.1\% \pm 1.8\%$, $34.3\% \pm 2.3\%$, and $60.1\% \pm 2.6\%$, respectively. No difference in fMHC expression was observed in the muscle myofiber. However, the expression of both sMHC and PGC-1α increased with time after cardiotoxin injury (Fig. 4A, B). The percentage of sMHC-expressing fibers increased from $46.9\% \pm 6.4\%$ 4 weeks after injury to $69.9\% \pm 1.9\%$ 8 weeks after injury, whereas the percentage of PGC-1α expressing fibers increased from $81.0\% \pm 6.1\%$ 4 weeks after injury to $94.7\% \pm 1.5\%$ 8 weeks after injury.

PGC-1α Expression in Different Types of Myofibers

Using immunohistochemistry, we examined the expression of PGC-1α protein in mouse soleus muscles to determine the relationship between PGC-1α protein levels and myofiber types in normal soleus muscles. Myofibers in the normal noninjured mouse soleus exhibited various levels of PGC-1α expression (Fig. 5B). There appeared to be no relationship between myofiber type and PGC-1α protein levels (Fig. 5B, C), since PGC-1α is expressed in both fast and slow muscle fibers.

PGC-1α protein expression was visible mainly in the centralized nucleus of regenerating myofibers (Fig. 5D; arrows), although the cytoplasm of some small regenerating myofibers stained uniformly for PGC-1α (Fig. 5E; asterisks). As shown in Figure 6A, B, D, E, and G, muscle fibers expressing fMHC, sMHC, and hybrid MHC also expressed PGC-1α at 2 and 4 weeks after injury. However, predominantly slow-type myofibers appeared to express PGC-1α at 8 weeks after injury (Fig. 6C, F, G).

DISCUSSION

Muscle injuries may occur as a result of traumas or other clinical causes, but the general process of muscle damage and repair is similar in most cases.[11–18] Muscle repair phases are: degeneration and inflammation, regeneration, and scar tissue formation.[12,14] The muscle regeneration cycle begins early at about 1 week after injury, peaking during the 2nd week, and then declining.[12,14,19] Muscle repair is a highly synchronized process involving the induction of various cellular

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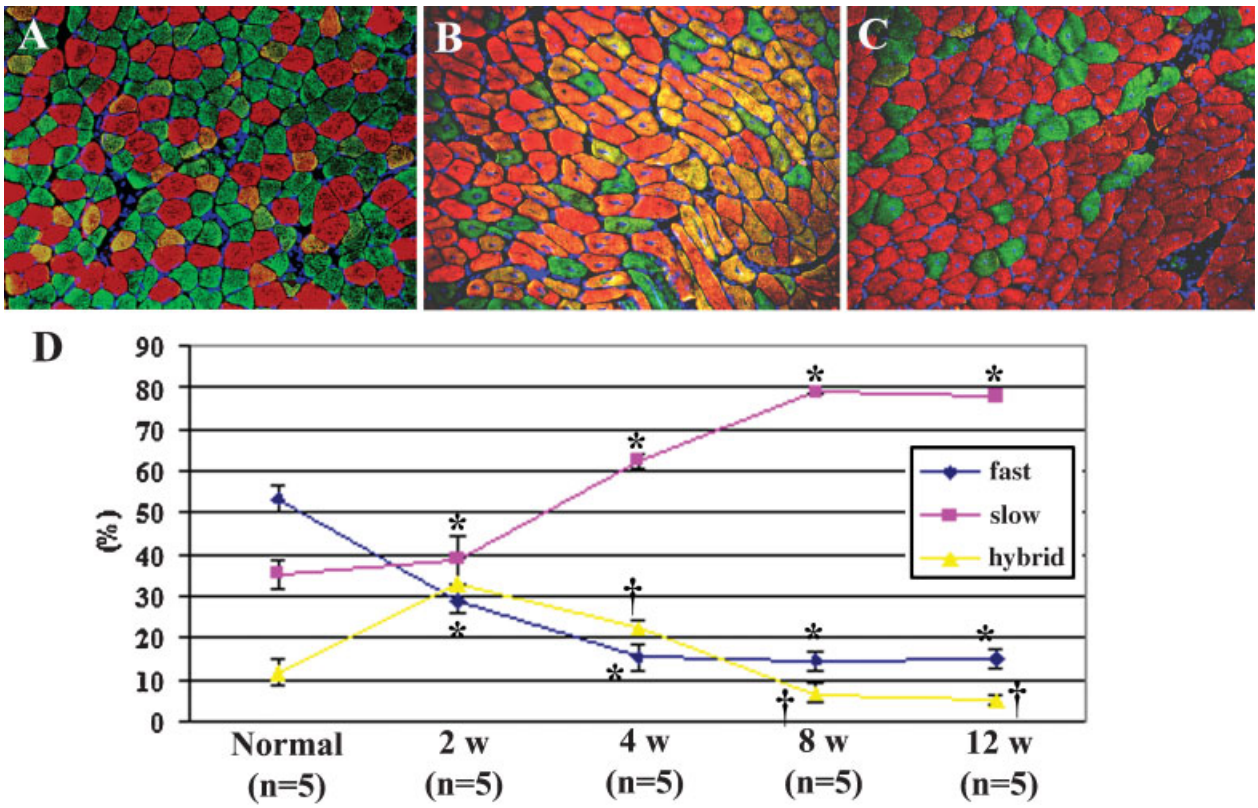

Figure 2. Immunohistochemical evaluation of the muscle fiber type conversion in cardiotoxin-injured muscle. (A) Representative images of sections of normal muscle, (B) 2 weeks postinjury, and (C) 4 weeks postinjury, respectively (original magnification, ×200). (D) The percentage of each fiber type observed in normal muscle and injured muscle 2, 4, 8, and 12 weeks after injury. *$P < 0.05$ compared with normal; †$P < 0.05$ compared with 2 weeks.

responses.[20] However, despite extensive research attempting to unravel the process of skeletal muscle repair, the complex regulatory pathways remain poorly understood.

Although researchers and clinicians typically regard the soleus as a slow muscle, our study found that 53% of the myofibers in the normal soleus muscles of the 5-week-old mice were fMHC-type myofibers, 35% were sMHC-type myofibers, and 12% were hybrid-type myofibers (Fig. 1). Our results parallel those previously reported by Farnbach et al., who used ATPase staining to demonstrate that normal soleus muscles from mice 1–3 months in age contained 53%–64% fast fibers and 36%–47% slow fibers.[21]

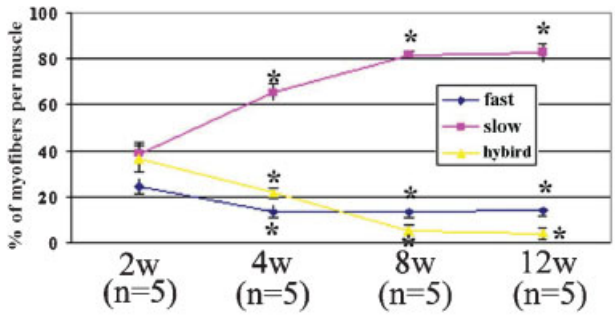

Figure 3. The percentage of centronucleated myofibers (newly regenerated fibers) that expressed fMHC-, sMHC-, or both fMHC and sMHC during muscle repair. *$P < 0.05$ compared with 2 weeks.

In this report, we found that 2 weeks after cardiotoxin injection, 33% of all muscle fibers in the injured soleus muscles were hybrid-type fibers; however, by 8 weeks after injury, this percentage had decreased to 6%. During that period, we observed an increase in the percentage of slow-type fibers and a concomitant decrease in the percentage of fast-type fibers in the injured muscle (Fig. 2). These findings are similar to those reported by Salvini et al.[7] Although there are subcategories of mouse fast-type fibers, we were unable to distinguish the different types because the fMHC antibody used in this study reacted with all subgroups. Thus the fMHC-type myofibers identified in our study likely included all fast-type fibers. Our analysis of postinjury,

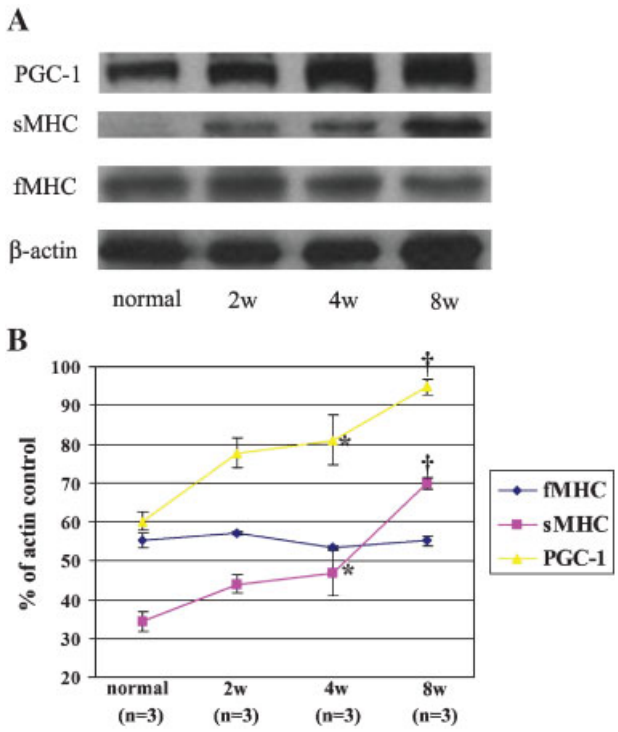

Figure 4. Western blot analysis of fMHC, sMHC, and PGC-1α protein expression (A). Quantitative analysis of western blot results demonstrated a time-dependent increase in the expression of sMHC and PGC-1α after cardiotoxin injury. In contrast, fMHC expression remained unchanged (B). $^{*}P < 0.05$ compared with normal; $^{\dagger}P < 0.05$ compared with 4 weeks.

regenerating myofibers also revealed an increase in the percentage of slow-type myofibers and decreases in the percentages of both fast- and hybrid-type myofibers (Fig. 3). Therefore, it is possible that cardiotoxin-induced injury leads muscle fibers to undergo conversion from fast to hybrid to slow fibers in a time-dependent manner.

Calcium signaling through calcineurin is the main regulatory pathway of slow fiber gene expression. Two of the known effectors of calcineurin signaling on gene expression in muscle are the Nfat and Mef2 transcription factor families. Using fiber-type-specific promoters, Lin et al.[8] showed that PGC-1α interacts directly with several Mef2 proteins in cultured muscle cells, but does not seem to co-activate the transcriptional activity of Nfatc3. We speculate that cooperation between PGC-1α and the calcineurin pathway may be important in the switching of muscle fiber type during the repair process, and a significant portion of this interaction may occur as a consequence of the direct co-activation of Mef2 proteins by PGC-1α. Additionally, fiber type conversion involves morphologic and biochemical changes that result in altered contractile properties and differing endurance capacities. Researchers have only begun to understand the cellular signaling mechanisms involved in muscle fiber type conversions.[22] PGC-1α seems to play an important role in the

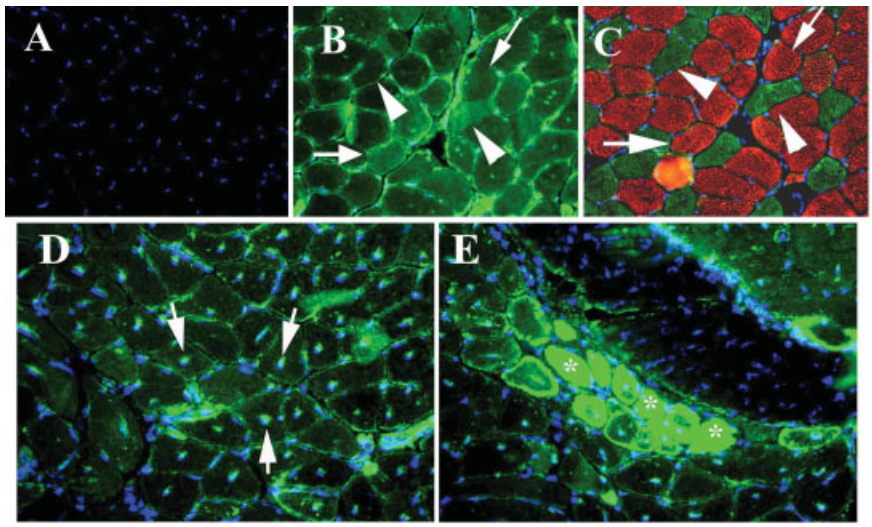

Figure 5. Immunohistochemical staining for PGC-1α (green; B), fMHC (green, arrowhead; C), and sMHC (red, arrow; C) in normal mouse soleus muscle (original magnification, ×400). In the negative control, PGC-1α was not visible (A). There was no discernible relationship between the distributions of fast and slow fibers and PGC-1α expression in normal mouse soleus. Immunohistochemical staining for PGC-1α in injured mouse soleus muscle is shown in (D) and (E) (original magnification, ×400). Four weeks after injury, PGC-1α protein was visible in the centralized nucleus of regenerating myofibers (arrow; D) and within the cytoplasm of some small regenerating myofibers (asterisk; E).

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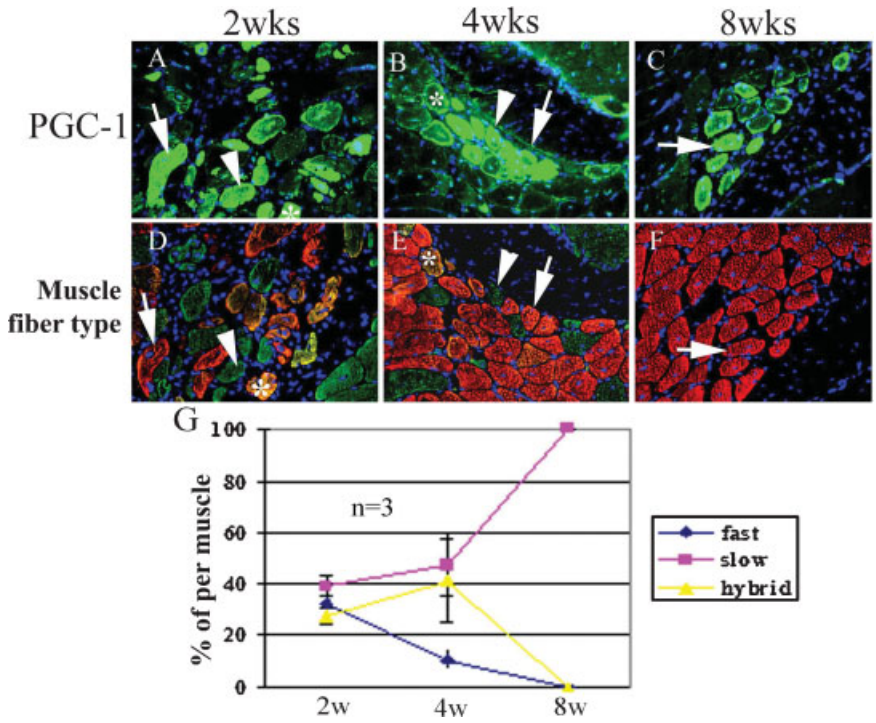

Figure 6. Immunohistochemical staining for PGC-1α (A–C) and fMHC (green, arrowhead), sMHC (red, arrow), and both fMHC and sMHC (yellow, asterisk) (D–F, respectively) during muscle repair (original magnification, ×400). Two and 4 weeks after injury, we observed PGC-1α expression in fast-, slow-, and hybrid-type myofibers (A, B, D, and E). However, 8 weeks after injury, PGC-1α appears to be expressed predominantly in slow fibers (C and F). (G) The percentage of each type of fiber that expressed PGC-1α at 2, 4, and 8 weeks after injury.

conversion of fast into slow myofibers.[9] Our western blot analysis showed that the expression of both PGC-1α and sMHC increased with time after cardiotoxin injection, whereas fMHC expression in the injured muscles remained similar to that in the noninjured muscles (Fig. 4). These results indicate that the increase in PGC-1α protein expression paralleled the increase in sMHC expression.

Lin et al. has demonstrated preferential expression of PGC-1α in the soleus muscle of mice. However, they did not examine the PGC-1α protein content in individual myofibers.[8] Russell et al. has reported that, in humans, PGC-1α protein content is highest in type IIa myofibers, lower in type I myofibers, and lowest in type IIx myofibers.[9] We did not find a relationship between fiber type and PGC-1α protein content in the skeletal muscle of normal mice (Fig. 5B, C). However, comparisons between the results from our study and the previously reported human studies should be made with care because of possible interspecific differences and different antibody manufacturers. The major finding pertaining to PGC-1α staining in our study is that we observed PGC-1α expression in the centralized nucleus of myofibers during muscle repair (Fig. 5D). These results suggest that PGC-1α represent a potent transcriptional co-activator for nuclear receptors,[23,24] and these are likely more active in the centralized nucleus early during muscle repair. As a principal regulator of slow fiber specification in mice,[8] PGC-1α, as we hypothesize, would trigger the formation of slow fibers. Our results show that the percentage of sMHC-expressing myofibers in regenerating muscle fibers increased with time after cardiotoxin injection (Fig. 3). As shown in Figure 6, PGC-1α protein expression was not fiber type specific 2 and 4 weeks after injury. However, predominantly slow myofibers expressed PGC-1α protein 8 weeks after injury. These facts suggest that PGC-1α expression during muscle repair might facilitate the conversion of fast myofibers into slow ones.

The results presented here show that PGC-1α plays an important role in the conversion of fast myofibers into slow myofibers during skeletal muscle repair. Future studies will investigate the relationship between functional recovery and fiber type conversion as well as PGC-1α expression during the repair of injured skeletal muscle.

ACKNOWLEDGMENTS

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Suramin can enhance the skeletal muscle healing by blocking myostation

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INTRODUCTION:

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although this type of injury is capable of healing, complete functional recovery is hindered by the development of scar tissue formation triggered by TGF-β1 [1]. We already reported suramin can effectively prevent the formation of fibrotic scar and block the proliferative effect of TGF-β1 on fibroblasts and can stimulate the differentiation on myogenic cells *in vitro* (unpublished data). Thus suramin can enhance muscle regeneration in the lacerated and strain-injured muscle [2, 3]. Furthermore we also reported blockade of myostatin (MSTN), a member of TGF-β super family, by decorin, other anti-fibrotic agent, showed enhancement of the fusion on myoblasts and inhibitory effect of fibroblast proliferation *in vitro* [4]. This finding brought us more interest to investigate another pathway of suramin to regulate fibroblasts and myoblasts by blocking the effect of MSTN. We performed this study to examine whether suramin would block MSTN's proliferative effect on fibroblasts and inhibitory effect of myoblasts differentiation *in vitro* and reduce the expression of MSTN in injured muscle to improve muscle healing *in vivo*, using an animal model of muscle contusion.

MATERIALS AND METHODS:

3T3 cell proliferation assay: 3T3 fibroblasts were cultured in 96 well plates (n=5) with DMEM containing 2% serum replacement (Sigma, St Louis, MO) and different concentration of MSTN (0 and 1 μg/ml) and suramin (0 and 50 μg/ml). Three days after incubation Cell Titer Cell Proliferation Assay kit (Promega, Madison, WI) was used to measure cell proliferation.

C2C12 cell differentiation assay: C2C12 myoblasts were cultured in 24 well plates (n=4) with differentiation medium (DMEM containing 2% horse serum and 1% penicillin/streptomycin) containing different concentration of MSTN (0 and 1 μg/ml) and suramin (0, 1, and 25 μg/ml). Three days after incubation immunocytochemistry of myosin heavy chain was done and the fusion index was assessed by counting the number of nuclei in differentiated myotubes as a percentage of the total number of nuclei.

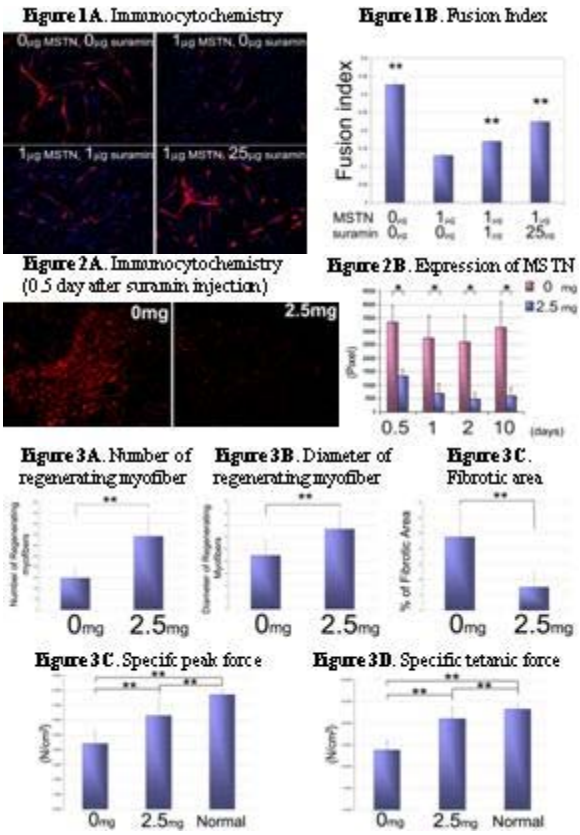
Animal model: The muscle contusion model was developed in tibialis anterior muscle of normal wild-type mice. Different concentrations of suramin (0 and 2.5 mg in 20 μl of Phosphate-buffered solution [PBS]) were injected intramuscularly two weeks after injury (five mice in each group). Cryostat sections of muscles were obtained and histologically stained (hematoxlin and eosin stain (H&E) and Masson's Trichrome stain) to evaluate the regeneration by counting the number of centronucleated regenerating myofibers and measuring fibrosis four weeks after injury. Specific peak force and specific tetanic force were mesured as physiological tests to evaluate functional recovery after muscle injury using same protocol as above (five mice in each group). Furthermore immunohistochemistry of MSTN was performed at different time point (0.5, 1, 2, 10 days after suramin (0 and 2.5 mg) injection) to evaluate the expression of MSTN (five mice in each group at each time point). Northern Eclipse software (Empix Image, Inc.) was used to quantify the total fibrotic area and expression of MSTN. Statistical analysis was performed with student's t-test or ANOVA. Statistical significance was defined as $P < .05$ (*: P<.05, **: P<.01).

RESULTS:

Suramin blocked the proliferative effect of MSTN on fibroblasts and the inhibitory effect of MSDN of myoblast differentiation: MSTN treatment significantly promoted the proliferation on fibroblasts and the differentiation on myoblasts. However, suramin treatment significantly blocked both of those MSTN's effects and moreover suramin treatment stimulated the fusion on myoblasts in a dose-dependent manner in the presence of MSTN (Fig. 1A, B).

Suramin inhibits the myostatin expression in injured skeletal muscle: Suramin (2.5 mg) injection 2 weeks after contusion injury effectively inhibited the expression of MSTN when compared with the control group (0 mg) at different time points (0.5, 1, 2, 10 days after suramin injection) (Fig. 2A, B).

Suramin enhances muscle regeneration and decreases fibrosis and improves functional recovery after contusion injury: We observed a significant increase in the number and in the diameter of regenerating myofibers in the suramin treated group (2.5 mg) when compared with the control group (0mg) (Fig. 3A, B,). Moreover suramin treated group showed significantly less fibrotic area than control group (Fig. 3C). Furthermore the suramin treatment showed significant advantage in physiological evaluation (specific peak force and specific tetanic force) compared to control group.



DISCUSSION:

We have reported that suramin can effectively prevent muscle fibrosis and enhance muscle regeneration by blocking TGF-β1 after laceration and strain injury [2, 3]. Nevertheless, whether suramin would regulate the effect of MSTN, negative regulator of muscle growth, to improve muscle healing was still unknown. This is the first study to show that suramin down-regulates the proliferation on fibroblasts and up-regulates the differentiation on myoblasts by neutralizing MSTN. Moreover suramin injection in injured skeletal muscle effectively inhibited the expression of MSTN and enhanced the muscle regeneration and reduced the fibrosis *in vivo*. These results may reveal the hidden mechanism by which suramin improve the muscle healing after injuries. We have reported important anti-fibrotic molecules such as Decorin to improve injured skeletal muscles. However suramin has been already approved by FDA and this makes suramin more suitable agent for the therapy of muscle injury. Our findings may contribute to the development of biological therapies for muscle injury.

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Improved Muscle Healing After Contusion Injury by the Inhibitory Effect of Suramin on Myostatin, a Negative Regulator of Muscle Growth

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Improved Muscle Healing After Contusion Injury by the Inhibitory Effect of Suramin on Myostatin, a Negative Regulator of Muscle Growth

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Background: Muscle contusions are the most common muscle injuries in sports medicine. Although these injuries are capable of healing, incomplete functional recovery often occurs.

Hypothesis: Suramin enhances muscle healing by both stimulating muscle regeneration and preventing fibrosis in contused skeletal muscle.

Study Design: Controlled laboratory study.

Methods: In vitro: Myoblasts (C2C12 cells) and muscle-derived stem cells (MDSCs) were cultured with suramin, and the potential of suramin to induce their differentiation was evaluated. Furthermore, MDSCs were cocultured with suramin and myostatin (MSTN) to monitor the capability of suramin to neutralize the effect of MSTN. In vivo: Varying concentrations of suramin were injected in the tibialis anterior muscle of mice 2 weeks after muscle contusion injury. Muscle regeneration and scar tissue formation were evaluated by histologic analysis and functional recovery was measured by physiologic testing

Results: In vitro: Suramin stimulated the differentiation of myoblasts and MDSCs in a dose-dependent manner. Moreover, suramin neutralized the inhibitory effect of MSTN on MDSC differentiation. In vivo: Suramin treatment significantly promoted muscle regeneration, decreased fibrosis formation, reduced myostatin expression in injured muscle, and increased muscle strength after contusion injury.

Conclusion: Intramuscular injection of suramin after a contusion injury improved overall skeletal muscle healing. Suramin enhanced myoblast and MDSC differentiation and neutralized MSTN's negative effect on myogenic differentiation in vitro, which suggests a possible mechanism for the beneficial effects that this pharmacologic agent exhibits in vivo.

Clinical Relevance: These findings could contribute to the development of biological treatments to aid in muscle healing after experiencing a muscle injury.

Keywords: muscle contusion injury; suramin; myostatin; muscle regeneration; fibrosis

Muscle injuries are common musculoskeletal problems encountered in sports medicine clinics. Muscle contusion, produced by the impact of a nonpenetrating object,[8] is one of the most common muscle injuries.[3] Current therapies including RICE (rest, ice, compression, and elevation), immobilization, as well as active and passive range of motion exercise are the norm for treatment; however, complications such as muscle atrophy, contracture formation, and pain leading to functional and structural deficits often occur after severe muscle injury.[8,24,25,38] Optimal treatment strategies have not yet been clearly defined.

We have observed that the healing process of injured skeletal muscle in animal models consists of 3 distinct phases:

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degeneration and inflammation, regeneration, and fibrosis.[13,15,16,26] The first phase, which occurs in the first few days after injury, is characterized by local swelling at the injury site, the formation of a hematoma, necrosis of muscle tissue,[20,23] degeneration, and an inflammatory response, which consists of the infiltration of activated macrophages and T-lymphocytes into the injured tissue. The next phase, regeneration, usually occurs 5 to 10 days after injury and includes phagocytosis of the damaged tissue and regeneration of the injured muscle. This phase is promoted by the release of several growth factors that regulate myoblast proliferation and differentiation, produce connective tissue and scar tissue, and induce capillary ingrowth at the injury site.[1,4,17]

The final phase, the formation of scar tissue (fibrosis), usually begins between the second and third week after injury. The formation of scar appears to be the end product of the muscle repair process and hinders full muscle regeneration. We have previously reported that transforming growth factor–beta 1 (TGF-β1) is a major factor in triggering the fibrotic cascade within injured skeletal muscle.[30,31] With this in mind, we have focused on the use of antifibrotic agents (such as decorin and γ-interferon) that inhibit TGF-β1 expression, reduce scar tissue formation, and consequently improve muscle healing after injury.[12,13] Administration of decorin after muscle injury showed improvement of muscle healing both histologically and physiologically.[13] The injection of γ-interferon into injured skeletal muscle also showed similar effects.[12] Despite these compelling findings, which were derived from various murine injury models, the fact that decorin is not clinically available limits its translation to human subjects and γ-interferon demonstrates serious side effects in spite of US Food and Drug Administration (FDA) approval. A recently published study has shown that myostatin (MSTN), a member of the TGF-β superfamily and a negative regulator of muscle growth, stimulates scar tissue formation after skeletal muscle injury in vivo.[48] It was also shown in this study that decorin could inhibit MSTN's activity in vitro.[48] Because both decorin and suramin have the ability to inhibit TGF-β1 activity,[5,6,13] we hypothesize that suramin may also possess a similar anti-MSTN activity as decorin.

Suramin, an antiparasitic and antineoplastic agent, can inhibit TGF-β1's ability to bind to its receptors and has been shown to enhance muscle regeneration after strain and laceration injuries.[5,6] We pursued the use of suramin due to the fact that it is currently FDA-approved and could thereby be used clinically more readily than decorin. In the current study, we examine whether suramin can promote differentiation of myoblasts and muscle-derived stem cells (MDSCs) and neutralize the effect of MSTN in vitro. We also investigate whether suramin can improve muscle healing after muscle contusion, a common muscle injury, by enhancing regeneration and reducing fibrosis in vivo.

MATERIALS AND METHODS

In Vitro Potential of Suramin to Induce Myogenic Differentiation

Effect of Suramin on C2C12 Myoblasts. C2C12 cells, a well-known myoblast cell line, were cultured with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, California) containing 10% fetal bovine serum (FBS) (Invitrogen), and 1% penicillin/streptomycin (P/S) (Invitrogen). Cells were plated at a density of 10 000 cells/well into 12-well plates. After a 24-hour incubation period, the medium was completely removed and low serum-containing medium (DMEM, 2% horse serum [HS] [Invitrogen], and 1% P/S) was added with varying concentrations of suramin (0, 0.25, 2.5, 25 μg/mL) (Sigma, St Louis, Missouri). Medium was replaced with fresh medium (containing the same concentrations of suramin) every 2 days. All the cells were grown at 37°C in 5% CO_2 for a total of 4 days.

Effect of Suramin on MDSCs Isolated From Skeletal Muscle. MDSCs were isolated from 3-week-old male mice (C57BL10J+/+) via the previously described modified preplate technique.[31,35,36] Muscle-derived stem cells were cultured in proliferation medium (PM) containing DMEM, 10% FBS, 10% HS, 1% P/S, and 0.5% chick embryo extract (Sera Laboratories International, West Sussex, United Kingdom). Cells were plated at a density of 10 000 cells/well into 12-well plates. After a 24-hour incubation period, the medium was completely removed and low-serum medium was added with different concentrations of suramin (0, 1, 10, 100 μg/mL) for the next 24 hours. Medium was replaced with fresh low-serum medium for 1 more day. All the cells were grown at 37°C in 5% CO_2 for a total of 3 days.

Ability of Suramin to Neutralize MSTN's Inhibition of MDSC Differentiation. Muscle-derived stem cells were cultured in PM and plated at a density of 5000 cells/well into 24-well plates. After a 24-hour incubation period, the medium was removed and low serum-containing medium was added with varying concentrations of suramin (0, 1, 10, or 100 μg/mL) and MSTN (0 or 100 ng/mL) for the next 24 hours. Medium was replaced with fresh low serum medium containing the same concentrations of MSTN for 1 additional day. All the cells were grown at 37°C in 5% CO_2 for a total of 3 days.

Immunocytochemistry

To quantify the differentiation of the C2C12 cells and MDSCs, cells were fixed in cold methanol for 2 minutes and washed in Dulbecco's phosphate-buffered saline (PBS) for 10 minutes at room temperature (RT). Samples were washed 3 times in PBS, then incubated in blocking buffer (10% HS) for 30 minutes at RT. Cells were incubated overnight at 4°C with primary antibodies (monoclonal antiskeletal myosin [fast] clone MY-32 [Sigma]) in 2% HS. After washing in PBS, samples were incubated with the secondary antibody (goat antimouse IgG conjugated with Cy3 [Sigma]) in 2% HS for 1 hour at RT. The cell nuclei were stained with a Hoechst 33258 dye for 10 minutes at RT. Fusion index (ratio of nuclei in myotubes to all nuclei) was calculated to evaluate the myogenic differentiation capacity of cells.

Evaluation of the Histologic and Physiologic Effects of Suramin on Muscle Healing After Contusion Injury In Vivo

Animal Model. The policies and procedures followed for the animal experimentation performed in these studies are

in accordance with those detailed by the US Department of Health and Human Services and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* Our Institutional Animal Care and Use Committee approved the research protocol used for these experiments (protocol No. 19/05). An animal model of muscle contusion was developed in normal mice (C57BL6J+/+, Jackson Laboratory, Bar Harbor, Maine) based on previously described studies.[8,26] Thirty-three mice, aged 8 to 10 weeks with weights of 21.0 to 26.3 g, were used in this experiment.

The mice were anesthetized with 1.0% to 1.5% isoflurane (Abbott Laboratories, North Chicago, Illinois) in 100% O_2 gas. The mouse's hindlimb was positioned by extending the knee and plantar flexing the ankle 90°. A 16.2-g, 1.6-cm stainless steel ball (Small Parts Inc, Miami Lakes, Florida) was dropped from a height of 100 cm onto the impactor that hit the mouse's tibialis anterior (TA) muscle. The muscle contusion made by this method was a high-energy blunt injury that created a large hematoma and was followed by massive muscle regeneration,[8,26] healing processes that are very similar to those seen in humans.[10] The mice were divided into 4 groups (6 mice/group) on the basis of the different concentrations of suramin to be injected (0, 2.5, 5, and 10 mg in 20 μL of PBS) after creating the contusion injury. Suramin was injected 2 weeks after injury. All animals were sacrificed to evaluate the healing histologically and physiologically at 4 weeks after injury. Three mice (6 muscles) per group were assessed histologically, 3 mice (6 muscles) per group were assessed physiologically, and 3 mice were used as normal controls in the physiologic tests.

Furthermore, 2 concentrations of suramin (0 and 2.5 mg in 20 μL of PBS) were injected 2 weeks after injury (3 mice/group). The mice of both groups were sacrificed 2 days after injection for histologic analysis of MSTN expression.

Evaluation of Muscle Regeneration After Suramin Therapy. Tibialis anterior muscles that were used for histologic evaluation were isolated and frozen in 2-methylbutane precooled in liquid nitrogen and subsequently cryosectioned. Hematoxylin and eosin staining was done to monitor the number of regenerating myofibers within the injury sites treated with suramin (0, 2.5, 5, or 10 mg in 20 μL of PBS), and the results were compared among the different groups. Centronucleated myofibers were considered to be regenerating myofibers. Centrally nucleated myofibers are a sign of regeneration in injured and diseased muscle. Upon myoblast fusing into myotubes or with myofibers, the nuclei remain in the endomysial tube found in the center of the myofiber until it has reached maturity whereby the nuclei migrate to their final positions at the periphery of the mature fiber in the subsarcolemmal position. The central nucleation is easily identified by hematoxylin and eosin or trichrome staining.[11,37] Analysis of regenerating myofibers was performed using Northern Eclipse software (Empix Imaging Inc, Cheektawaga, New York). The total number of regenerating myofibers within the injury site was quantified by using 10 random fields selected from each sample in accordance with a previously described protocol.[12,13,26,33]

Evaluation of Fibrosis After Suramin Therapy. To measure areas of fibrotic tissue in the injury sites, Masson's trichrome staining (IMEB Inc, Chicago, Illinois) was performed. After Masson trichrome staining, the ratio of the fibrotic area to the total cross-sectional area was calculated to estimate the fibrosis formation by using Northern Eclipse software (Empix Imaging Inc). The ratio of the fibrotic area within the injury sites was quantified using a previously described protocol.[12,13,26, 3]

Evaluation of MSTN Expression After Suramin Therapy. To measure the MSTN expression in injured muscle, cryosectioned tissue was fixed in 4% formalin for 5 minutes followed by two 10-minute washes with PBS. The sections were then blocked with 10% HS for 1 hour at RT. Sections were incubated overnight at 4°C with primary antibodies (goat anti GDF-8 AF-788, R&D Systems Inc, Minneapolis, Minnesota) in 2% HS. After washing in PBS, samples were incubated with the secondary antibody (donkey anti-goat IgG conjugated with Alexa Fluor 555 [Invitrogen]) in 2% HS for 1 hour at RT.

The total MSTN positive area was calculated using Northern Eclipse software (Empix Imaging Inc) The total positive area within the injury site was quantified using a previously described protocol.[12,13,26,33]

Physiologic Evaluation of Muscle Contractile Properties After Suramin Therapy. Contusion injuries were made on TA muscles of both legs of normal mice and treated as described above. Four weeks after injury, muscles from both legs were tested to evaluate peak twitch and tetanic force. Tibialis anterior muscles were harvested bilaterally, placed in a vertical chamber that was constantly perfused with mammalian Ringer solution aerated with 95% O_2–5% CO_2 and maintained at 25°C. The distal attachment of the muscle was mounted to a glass tissue-support rod, and the proximal end of muscle on the tibia was connected to a force transducer and length servo system (Aurora Scientific, Aurora, Ontario, Canada). The muscles were stimulated by monophasic rectangular pulses of current (1 millisecond in duration) delivered through platinum electrodes placed 1 cm apart. The current was increased by 50% more than the current necessary to obtain peak force (250-300 mA) to ensure maximal stimulation. Using a micropositioner, muscles were first adjusted to their optimum length (Lo), defined as the length at which maximum isometric twitch tension was produced. Maximal tetanic force was assessed via a stimulation frequency of 75 Hz delivered in a 500-millisecond train. After the procedure, each muscle was weighed and specific peak twitch force and specific peak tetanic force were calculated and expressed in force per unit cross-sectional area (N/cm^2).

Statistical Analysis

All of the results from this study were expressed as the mean ± standard deviation. The result of MSTN expression in vivo was analyzed using the *t* test and all the other results were statistically analyzed using analysis of variance. Differences among the groups were analyzed by using Scheffé multiple comparisons (post hoc test). Statistical significance was defined as $P < .05$.

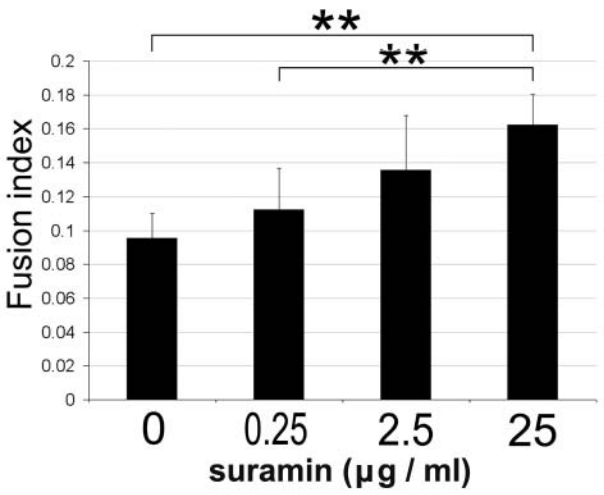

Figure 1. Fusion index was measured to evaluate C2C12 myoblast differentiation stimulated by different concentration of suramin (0, 0.25, 2.5, and 25 µg/mL). $^{**}P < .01$.

RESULTS

In Vitro Potential of Suramin to Induce Myogenic Differentiation

Effect of Suramin on Myoblasts. The effect of suramin on the differentiation of C2C12 cells is shown in Figure 1. The fusion index of C2C12 cells in the group treated with suramin (25 µg/mL) (0.162 ± 0.018) was significantly higher at 4 days of incubation as compared with the fusion index in the control group (0 µg/mL) (0.953 ± 0.015). Moreover, the high-dose suramin-treated group (25 µg/mL) displayed a significantly higher fusion index than the low-dose suramin-treated group (0.25 µg/mL). The effect of suramin to induce the differentiation of C2C12 appeared to be dose-dependent.

Effect of Suramin on MDSCs. Suramin also stimulated MDSC differentiation in a dose-dependent manner (Figure 2E). Suramin-treated MDSCs enhanced the differentiation of MDSCs after 3 days of incubation. Suramin-treated groups (10 and 100 µg/mL) (Figures 2C and 2D) showed significantly higher fusion indexes (0.228 ± 0.025, 0.347 ± 0.0456) than the control group (0 µg/mL) (Figure 2A) (0.149 ± 0.030). Among the suramin-treated groups, the high-dose suramin-treatment group (100 µg/mL) (Figure 2D) displayed a significantly higher fusion index when compared with the 2 lower-dose treatment groups (1 and 10 µg/mL) (Figures 2B and 2C).

Ability of Suramin to Neutralize MSTN's Inhibition of MDSC Differentiation. The group containing only MSTN (0 µg/mL suramin and 100 ng/mL MSTN) (Figure 3B) showed a significantly lower fusion index (0.091 ± 0.020) than the control group (0 µg/mL suramin and 0 ng/mL MSTN) (Figure 3A) (0.152 ± 0.035). All groups containing both suramin and MSTN (1, 10, and 100 µg/mL suramin and 100 ng/mL MSTN) (Figures 3C, 3D, and 3E) demonstrated significantly higher fusion indexes (0.153 ± 0.035, 0.162 ± 0.048, 0.279 ± 0.041) when compared with the group containing only MSTN (Figure 3B). Suramin appeared to neutralize MSTN's inhibitory effect on MDSC differentiation and, moreover, appeared to stimulate the fusion of MDSCs in a dose-dependent manner (Figure 3F).

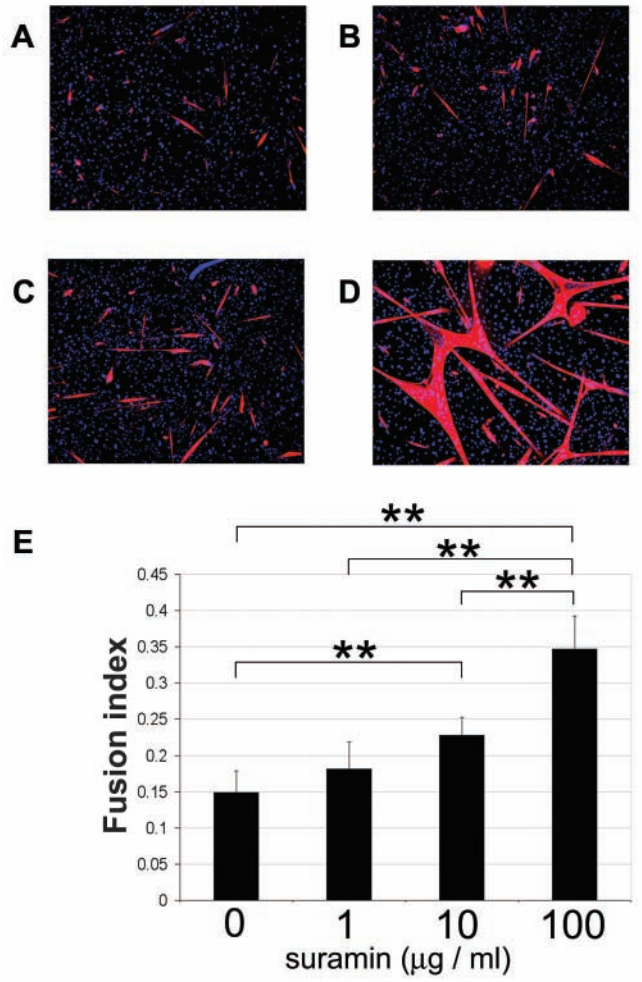

Figure 2. Immunocytochemical staining of muscle-derived stem cells (MDSCs) for fast myosin heavy chain at 3 days after incubation with different doses of suramin (A, 0 µg/mL; B, 1 µg/mL; C, 10 µg/mL; and D, 100 µg/mL). Myotubes are shown in red and nuclei are in blue (original magnification, ×200). E, comparison of fusion index of MDSC differentiation. $^{**}P < .01$.

Histologic and Physiologic Effects of Suramin on Muscle Healing After Contusion Injury In Vivo

Effect of Suramin Treatment on Muscle Regeneration After Contusion Injury. All the centronucleated regenerating myofibers present in the injured muscle were counted and compared among the groups (Figure 4E). The suramin-treated muscles displayed numerous regenerating myofibers at the contusion site. All the suramin-treated groups (2.5, 5, and 10 mg) (Figures 4B, 4C, and 4D) showed significantly higher numbers of regenerating myofibers (361.6 ± 84.39, 341.1 ± 57.21, 308.5 ± 84.33)

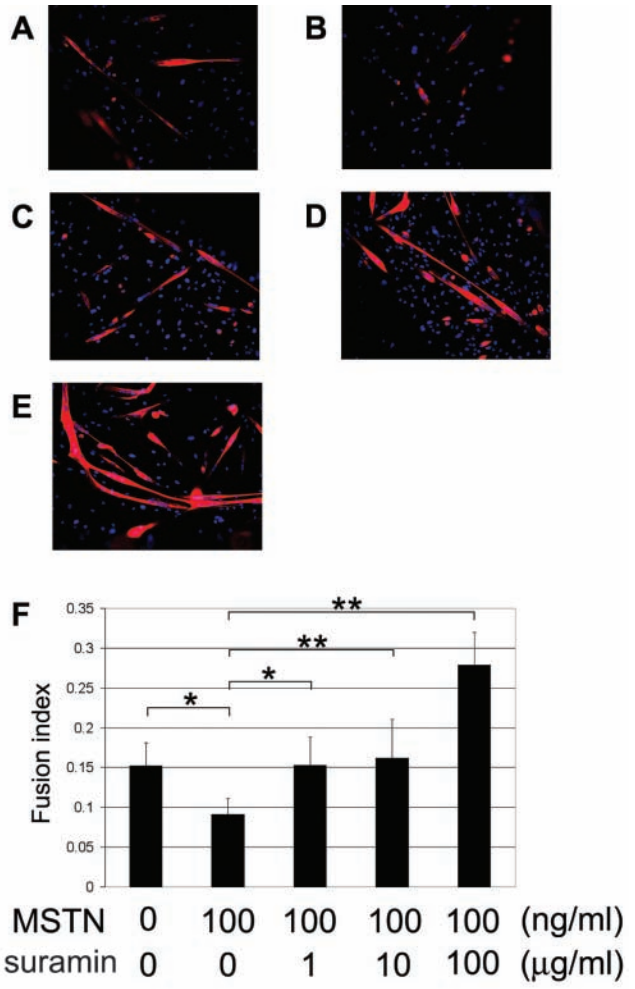

Figure 3. Immunocytochemical staining of muscle-derived stem cells (MDSCs) for fast myosin heavy chain at 3 days after incubation in 2% Dulbecco's modified Eagle's medium with different doses of suramin and myostatin (MSTN) (A, 0 μg/mL suramin and 0 ng/mL MSTN; B, 0 μg/mL suramin and 100 ng/mL MSTN; C, 1 μg/mL suramin and 100 ng/mL MSTN; D, 10 μg/mL suramin and 100 ng/mL MSTN; and E, 100 μg/mL suramin and 100 ng/mL MSTN). Myotubes are shown in red and nuclei are in blue (original magnification, ×200). F, comparison of fusion index of MDSC differentiation. *$P < .05$, **$P < .01$.

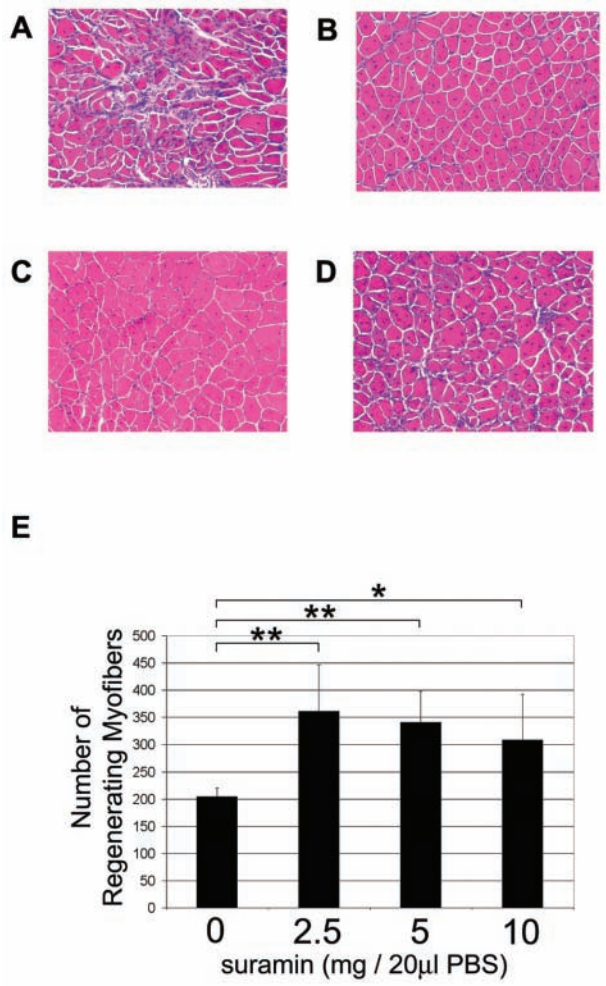

Figure 4. Histologic evaluation of muscle regeneration at four weeks after contusion injury by hematoxylin and eosin staining of tibialis anterior muscle treated with different concentrations of suramin (A, 0 mg/20 μL phosphate-buffered saline [PBS]; B, 2.5 mg/20 μL PBS; C, 5 mg/20 μL PBS; and D, 10 mg/20 μL PBS) injected at 2 weeks after injury. Regenerating myofibers were defined by centronucleated myofibers (original magnification, ×100). E, quantification of the number of regenerating myofibers treated with various concentrations of suramin. *$P < .05$, **$P < .01$.

compared with the control group (0 mg of suramin) (Figure 4A) (204.6 ± 15.27).

Effect of Suramin Therapy on Muscle Fibrosis After Contusion Injury. After Masson trichrome staining, the area of fibrotic scar tissue was evaluated and compared among the groups (Figure 5E). All the suramin-treated groups (2.5, 5, and 10 mg) (Figures 5B, 5C, and 5D) showed significantly less fibrotic area (6.856 ± 2.588, 7.677 ± 2.897, 8.993 ± 2.980) compared with the untreated control group (0 mg of suramin) (Figure 5A) (19.109 ± 3.215).

Suramin Injection Downregulated MSTN Expression in Injured Muscle. Immunohistochemical staining was performed to detect MSTN expression in the contusion-injured TA muscles. The MSTN-positive areas were measured and compared between the various treatment groups (Figure 6C). The muscle treated with 2.5-mg suramin injection 2 weeks after injury showed significantly less MSTN expression (4466.7 ± 7306.1) when compared with the untreated control (0 mg suramin) (27 830.2 ± 23 206.3) (Figures 6A and 6B).

Suramin Injection Improved Muscle Strength After Contusion Injury. The results of the physiologic evaluations are shown in Table 1. The control group (0 mg of suramin) and all the suramin-treatment groups, with the exception of the 2.5-mg suramin-treatment group, showed significantly less specific peak force (twitch and tetanic) when compared with the normal noninjured group. On the other hand, the muscles treated with 2.5 mg of suramin

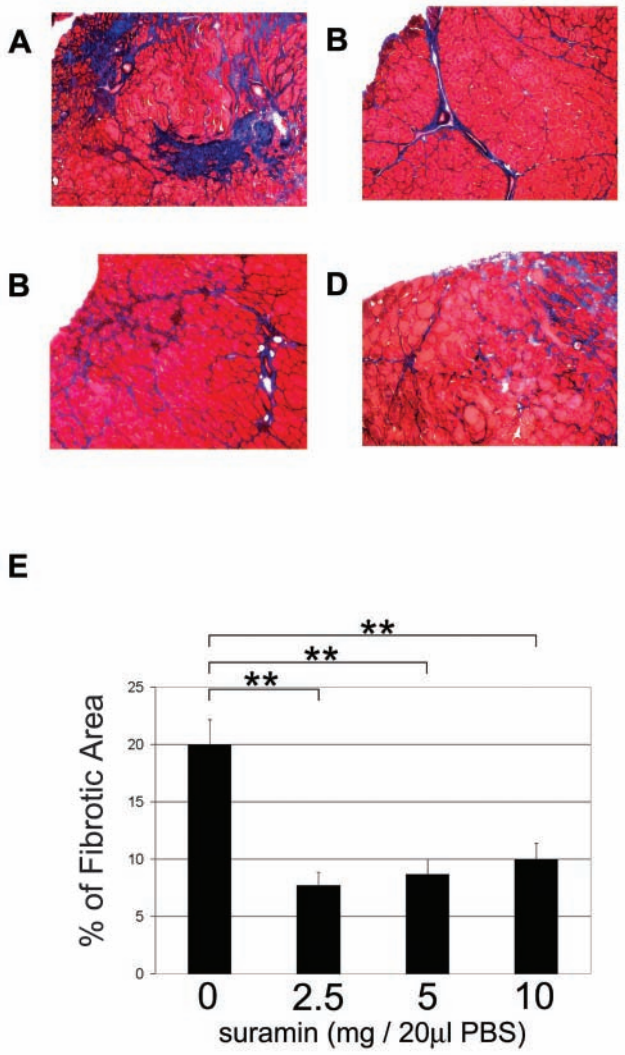

Figure 5. Histologic evaluation of the formation of scar tissue at 4 weeks after contusion injury by Masson trichrome staining of tibialis anterior muscle treated with different concentrations of suramin (A, 0 mg/20 µL phosphate-buffered saline [PBS]; B, 2.5 mg/20 µL PBS; C, 5 mg/20 µL PBS; and D, 10 mg/20 µL PBS) injected at 2 weeks after injury. Scar tissues are shown in blue and muscles are in red (original magnification, ×100). E, quantification of the scar tissue area in tibialis anterior muscle treated with various concentrations of suramin. $^{**}P < .01$.

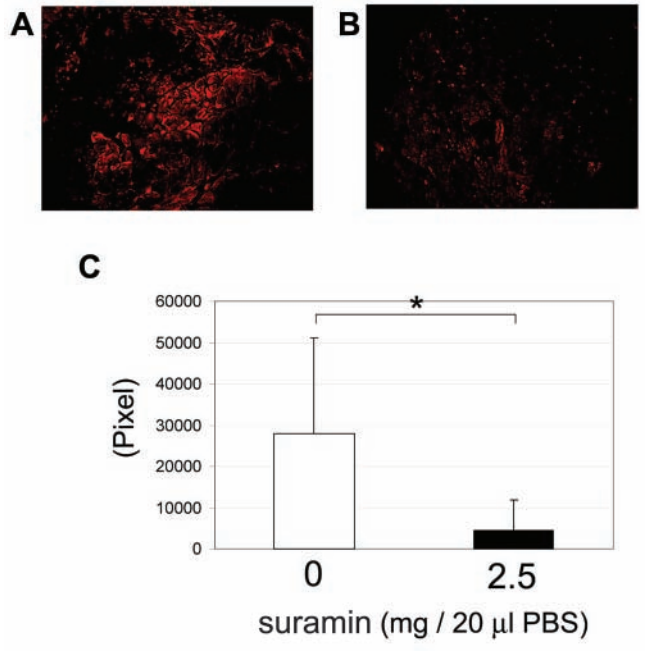

Figure 6. Immunohistochemical staining for MSTN at 2 days after suramin injection 2 weeks after injury (A, 0 mg suramin/20 µL phosphate-buffered saline [PBS]; B, 2.5 mg suramin/20 µL PBS). C, quantification of the total MSTN-positive area treated with suramin. $^{*}P < .05$.

TABLE 1
Results of Specific Peak Twitch and Titanic Force

Group	Specific Peak Twitch Force (N/cm^2)	Specific Peak Titanic Force (N/cm^2)
Normal (noninjured)	7.728 ± 0.312^a	23.272 ± 1.334^a
0 mg suramin	4.431 ± 0.903	13.797 ± 2.136
2.5 mg suramin	6.305 ± 1.239^b	21.055 ± 2.662^a
5 mg suramin	5.014 ± 0.337	15.131 ± 0.830
10 mg suramin	4.993 ± 1.074	15.085 ± 2.792

$^a P < .01$ compared with 0, 5, and 10 mg suramin groups.
$^b P < .05$ compared with 0 mg suramin group.

showed significantly greater specific peak force (twitch and tetanic) than the untreated control group. There was no significant difference between injured muscle that had been treated with 2.5 mg of suramin and normal noninjured muscle. In addition, the muscles injected with 2.5 mg of suramin also showed significantly greater specific peak tetanic force than the other suramin-treated groups (5 and 10 mg) and the control group (0 mg of suramin).

DISCUSSION

The aim of this study was to evaluate the effect of suramin on myogenic cell differentiation and muscle healing after contusion injury. We hypothesized that suramin treatment would lead to better biological healing by both stimulating muscle regeneration and preventing fibrosis in contused muscle. Histologically, the muscle treated with suramin 2 weeks after injury showed more regenerating myofibers and less fibrotic scar formation when compared with the control group (0 mg of suramin) at 4 weeks after contusion injury. Furthermore, suramin treatment also showed an increase in muscle strength compared with the untreated control group.

The muscle contusion injuries we modeled in this study are among the most common muscle injuries encountered in contact sports and by military personnel. More than

90% of muscle injuries are caused either by contusion or by excessive strain of the muscle.[3,14] Although contusion injury is capable of healing, incomplete functional recovery often occurs, depending on the severity of the initial trauma. Skeletal muscle has a great regenerative potential,[†] largely attributed to the activation of muscle progenitor cells and their fusion into mature multinucleated myofibers[22,40,41]; however, scar tissue formation occurs simultaneously and likely competes with muscle regeneration during the muscle-healing process.[12,26,32]

We have investigated the effect of some treatments, such as suture or immobilization, which are normally used in the clinic for muscle injury, by using animal models. One study showed that immobilization after muscle laceration had no significant effect on fibrosis reduction. Suture repair promoted better healing of the injured muscle and prevented the development of fibrosis at the deep-tissue level, but not superficially.[33]

Suramin, which is a polysulphonated naphthylurea, was designed as an antiparasitic drug[18] and is used for the treatment of human sleeping sickness, onchocerciasis, and other diseases caused by trypanosomes and other worms. In addition, it is under investigation as a treatment for some malignancies such as prostate, adrenal cortex, lymphoma, breast, and colon cancers[49] and for human immunodeficiency virus–1.[29] The major systemic side effects of suramin are malaise, neuropathy,[2,7] mineral corticoid insufficiency,[27] and corneal deposits[19]; occasionally neutropenia,[9] thrombocytopenia,[47] and renal failure[42] have also been observed. The toxicity of suramin when injected intramuscularly, as we did in our animal model, has not yet been determined. Although we observed no adverse effects in the mice injected with up to 10 mg of suramin in this study, we did not specifically test to evaluate side effects. Suramin is known as a heparin analog that can bind to heparin-binding proteins and inhibit the effect of growth factors by competitively binding to growth factor receptors.[43,49] Transforming growth factor-β1, -β2, and -β3; platelet-derived growth factor (PDGF) A and B; and epidermal growth factor are growth factors that are known to be inhibited by suramin. Among them, TGF-β1 and -β2 and PDGF A and B are known to have the potential to promote fibroblast proliferation.[44,46]

In our in vivo study using the muscle-contusion model, direct injection of suramin at 2 weeks after muscle injury demonstrated a significant reduction of fibrous tissue formation when compared with the untreated control (0 mg of suramin). We found that suramin treatment led to a beneficial effect in contused muscle, as was previously seen in suramin-treated lacerations and strains[5,6] as well as when using other antifibrotic agents such as decorin and interferon gamma.[12,13] Moreover, our in vivo results indicate that 2.5 mg of suramin was the optimal dosage for the treatment of muscle contusions as there was no significant difference observed when higher dosages (5 and 10 mg) of suramin was injected into the injury site. Our previous work with the laceration and strain models compared doses that were different than the current study and we have difficulty making direct comparisons between the current study and the former studies. In the laceration study, we compared suramin doses at 0.25, 1.0, and 2.5 mg and found that the 2.5-mg dose was optimal.[6] In the strain model study, we compared doses at 0.25, 1.0, and 5 mg and found that the 5-mg dose was optimal.[5] Because we did not use a 2.5-mg dose in the strain study and did not use a 5-mg dose in the laceration study, we cannot definitively say that the 2.5-mg dosage would be optimal for these types of injuries, although it is strongly inferred.

This study also showed that suramin enhanced muscle regeneration when it was injected directly into the muscle 2 weeks after receiving a contusion injury. We found many regenerating myofibers at the injury site, usually sequestered by a large amount of fibrotic tissue. All the suramin-treated groups showed a significant increase in the number of regenerating myofibers at 4 weeks after receiving the injury.

Functional recovery after muscle injury is the most important variable determining the likelihood for clinical translation of this therapy in the treatment of skeletal muscle injury. As was observed with other antifibrotic agents (decorin and interferon gamma),[12,13] suramin also appears to have a beneficial effect on the physiologic recovery of skeletal muscle. Our results showed that there was no significant difference in the specific peak twitch and tetanic forces between normal noninjured muscle and contused 2.5-mg suramin-treated muscle. These results strongly indicate that the injection of 2.5 mg of suramin at 2 weeks after contusion injury can improve muscle strength and promote functional recovery after muscle injury.

Taken together, our in vitro study about the effect of suramin on myogenic cell differentiation provides insight as to the underlying mechanism by which suramin enhances muscle regeneration in vivo. Our in vitro results showed 25 μg/mL of suramin can significantly enhance C2C12 differentiation at 4 days after incubation and, in addition, suramin treatment leads to significant increases in the fusion index in a dose-dependent manner. Further, we observed an even more prominent dose-dependent effect on MDSC differentiation 3 days after initiating the incubation with suramin. Moreover, suramin also showed a neutralizing effect on MSTN, which inhibits differentiation of MDSCs in a dose-dependent manner.

We found that suramin stimulates myogenic differentiation in vitro. It is consistent with our in vivo results, showing that suramin is capable of enhancing muscle regeneration and improving muscle healing after muscle injury. Furthermore, as expected, we observed that MSTN significantly inhibits the myogenic differentiation of MDSCs; however, in the presence of suramin, the inhibitory effect of MSTN on myogenic differentiation was attenuated in a dose-dependent manner. These results suggest that suramin can neutralize MSTN activity. Therefore, we hypothesize that when myoblasts and MDSCs are treated with suramin, suramin stimulates myogenic differentiation by neutralizing the effect of endogenous MSTN. A similar neutralizing effect on MSTN has been seen when cells were treated with decorin, a TGF-β1 blocker.[48]

[†]References 4, 14, 16, 21, 26, 28, 33, 45

On the basis of these results, we further investigated whether suramin exerts a beneficial effect in the injured muscle through regulating MSTN expression. Not surprisingly, it was revealed that treatment with suramin significantly decreased MSTN expression in the injured muscle. Our previous study showed that MSTN can act with TGF-β1 to magnify fibrosis cascades in injured muscles.[48] Taken together, it may suggest that suramin administration effectively leads to enhanced muscle regeneration and reduced fibrosis after muscle injury via downregulation of endogenous MSTN.

This is the first study conducted to evaluate the effects that suramin would have on a contusion injury. It is also the first study to demonstrate that one of the mechanisms by which suramin could enhance skeletal muscle healing was by the downregulation of MSTN. Our results showed suramin can enhance myogenic cell differentiation and neutralize the effects of MSTN, which downregulates MDSC differentiation. Moreover, these in vitro results may reveal a possible mechanism by which suramin directly enhances muscle regeneration after muscle injury. Future studies should investigate further the mechanism by which suramin appears to stimulate myogenic differentiation and promotes muscle healing via MSTN regulation.

In summary, we demonstrated that the direct injection of suramin at 2 weeks after contusion injury can effectively reduce fibrotic scar formation and enhance muscle regeneration 4 weeks after injury. Physiologic evaluation also showed that suramin can enhance muscle functional recovery after contusion injury. Our in vitro study demonstrated that culturing either C2C12 myoblasts or MDSCs with suramin led to a significant increase in the fusion index of the cells. Moreover, with the addition of both suramin and MSTN in cell cultures, suramin was able to counteract MSTN's biological activity, thereby rescuing MSTN-inhibited myogenic differentiation of MDSCs.

The greatest advantage of using suramin is that this drug has already been approved by the FDA.[34,39] Future studies should consider the use of this agent for off-label use in the treatment of skeletal muscle injuries. Our findings could contribute to the development of progressive therapies for treating skeletal muscle injuries.

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Angiotensin II Receptor Blockade Administered After Injury Improves Muscle Regeneration and Decreases Fibrosis in Normal Skeletal Muscle

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Background: Several therapeutic agents have been shown to inhibit fibrosis and improve regeneration after injury in skeletal muscle by antagonizing transforming growth factor-β1. Angiotensin receptor blockers have been shown to have a similar effect on transforming growth factor-β1 in a variety of tissues.

Hypothesis: Systemic treatment of animals after injury of skeletal muscle with an angiotensin receptor blocker may decrease fibrosis and improve regeneration, mainly through transforming growth factor-β1 blockade, and can be used to improve skeletal muscle healing after injury.

Study Design: Controlled laboratory study.

Methods: Forty mice underwent bilateral partial gastrocnemius lacerations. Mice were assigned randomly to a control group (tap water), a low-dose angiotensin receptor blocker group (losartan, 0.05 mg/mL), or a high-dose angiotensin receptor blocker group (0.5 mg/mL). The medication was dissolved in tap water and administered enterally. Mice were sacrificed 3 or 5 weeks after injury, and the lacerated muscles were examined histologically for muscle regeneration and fibrosis.

Results: Compared with control mice at 3 and 5 weeks, angiotensin receptor blocker–treated mice exhibited a histologic dose-dependent improvement in muscle regeneration and a measurable reduction in fibrous tissue formation within the area of injury.

Conclusion: By modulating the response to local and systemic angiotensin II, angiotensin receptor blocker therapy significantly reduced fibrosis and led to an increase in the number of regenerating myofibers in acutely injured skeletal muscle. The clinical implications for this application of angiotensin receptor blockers are potentially far-reaching and include not only sports- and military-related injuries, but also diseases such as the muscular dystrophies and trauma- and surgery-related injury.

Clinical Relevance: Angiotensin receptor blockers may provide a safe, clinically available treatment for improving healing after skeletal muscle injury.

Keywords: fibrosis; angiotensin receptor blocker; TGF-β; muscle injury; muscle regeneration

Skeletal muscle injuries are among the most common complaints of patients seen by general medical physicians and also account for a large majority of patients seen in orthopaedic clinics.[2] Investigations have demonstrated that the natural history of muscle injury proceeds through a highly coordinated sequence of steps. Unfortunately, the regenerative capacity of injured skeletal muscle is limited; fibrotic tissue is a common end result that predisposes the muscle to recurrent injury and limits recovery of function.[16] Clinical experience reveals a high recurrence rate of skeletal muscle strain injuries among athletes, approaching 30% in some professional-level athletes.[32] Advancements in the identification of molecular events and cellular transformations after muscle injury have flourished; however, the clinical treatment of this common condition still relies on conventional therapies of rest, ice, and anti-inflammatory medications; these are of limited efficacy in preventing or

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treating posttraumatic muscle fibrosis and in reducing the rate of reinjury.[1,18,33]

In our laboratory, we have investigated several biological agents that have proved to be of some benefit in altering the natural course of muscle injury. Specifically, we have focused our recent efforts on agents that inhibit muscle fibrosis via inhibition of transforming growth factor-β1 (TGF-β1), a key cytokine in the fibrotic signaling pathway in skeletal muscle.[21] Using decorin, suramin, relaxin, and gamma interferon, we have demonstrated that these therapies can decrease fibrosis and increase regeneration after skeletal muscle injury.[9,13,14,22,24] However, their use clinically is hampered by relatively severe side-effect profiles, lack of oral dosing formulations, and in some cases, lack of Food and Drug Administration (FDA) approval for use in humans.

Fibrosis is a pathologic process that is not unique to the skeletal muscle system. Observations have linked pathologic fibrosis in various organ systems to the local effects of a naturally occurring molecule, angiotensin II, an end product of the blood pressure–regulating renin-angiotensin system. The modulation of angiotensin II with angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers has demonstrated decreased fibrosis and improved function in liver, kidney, and lung tissue.[23,27,28,30] Injured cardiac muscle in disease entities such as congestive heart failure also demonstrates dysfunction related to fibrosis. Myocardium exposed to decreased levels of angiotensin II, either through the use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (ARB), has also demonstrated measurably improved function.[15,31] Investigators have also observed a relationship between the modulation of angiotensin II and skeletal muscle. Patients treated with angiotensin-modulating medications for hypertension also displayed the unexpected side effect of decreased rates of muscle wasting and a reduction in the relative amount of adipose tissue within their musculature.[25] Moreover, elite athletes, particularly those in endurance sports, have also demonstrated findings consistent with inherent differences in their body's metabolism of angiotensin II, with decreased exposure resulting in improved skeletal muscle function.[25] We have observed an improvement in skeletal muscle regeneration postinjury after treatment with angiotensin blockade; this is a finding that, to our knowledge, had not been reported previously.[4] Elegant experiments have subsequently been reported that elucidate the mechanism by which angiotensin II receptor blockade modulates TGF-β1, which has also been implicated in the prevention of muscle regeneration in murine models of chronic myopathic disease.[10] The hypothesis of this study is that systemic delivery of an angiotensin II receptor blocker can enhance muscle regeneration and decrease fibrosis in a laceration model of normal skeletal muscle injury. This therapy may be an effective treatment when instituted postinjury, which is a clinically relevant scenario for physicians treating muscle injuries.

MATERIALS AND METHODS

In Vitro

The role of angiotensin II regarding myoblast and fibroblast proliferation was investigated using a series of standard proliferation experiments. National Institutes of Health (NIH) 3T3 cells (fibroblast cell line) and C2C12 myoblasts, purchased from American Type Culture Collection (ATCC) (Rockville, Maryland), along with primary muscle fibroblast isolates (PP2 cells) obtained through the previously published preplating technique, were chosen for analysis.[29] These cells were cultured with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, California) containing 10% fetal bovine serum, 10% horse serum, 0.5% chicken embryo extract, and 1% penicillin/streptomycin. All cells were cultured at 37°C in 5% CO_2. National Institutes of Health 3T3, C2C12, and PP2 cells were seeded onto different 6-well plates. Cells were cultured with medium (described above) and 0, 10^{-8}, 10^{-6}, or 10^{-4} mol/L of human angiotensin II (Sigma Chemical, St Louis, Missouri). Cell counts were performed at 24, 48, and 96 hours using a standard cell-counting chamber.

The supernatant solution from the PP2 cell culture was extracted after 24 and 48 hours of exposure to the various concentrations of angiotensin II. An enzyme-linked immunosorbent assay (ELISA) was performed in accordance with the manufacturer's recommendations (Sigma Chemical) to determine the level of TGF-β1 in the solution. National Institutes of Health 3T3 cells were harvested 48 hours after incubation with or without angiotensin II, and a western blot was performed. After lysation, the samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes used for immunostaining. Mouse anti-TGF-β1 type I antibodies (Sigma Chemical) were then applied. The horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, Illnois) were developed using SuperSignal West Pico Chemiluminescent substrate (Pierce), and positive bands were visualized on radiographic film. All results were analyzed using Northern Eclipse software v.6.0 (Empix Imaging, Mississauga, Ontario, Canada).

Animal Model

Forty immunocompetent mice (C57Bl/6J; Jackson Laboratory, Bar Harbor, Maine) were used for the histologic analysis. The animals were housed in cages and fed with commercial pellets. The policies and procedures of the animal laboratory were in accordance with those detailed by the US Department of Health and Human Services and the Animal Research Care Committee of the authors' institution (Protocol #25-03). A previously reported muscle laceration injury model was employed for these experiments, the healing process of which is similar histologically to strain injury models, which account for the most common clinical pattern of injury.[9,14,17,19,21] The mice were anesthetized with 16 μL of ketamine and 8 μL of xylazine in 25 μL of phosphate-buffered saline by intraperitoneal injection. For histologic analysis, lacerations were performed bilaterally on the gastrocnemius muscle through 50% of its width and 100% of its thickness at 60% of its length from its insertion. All mice were randomly assigned to 1 of 3 groups: (i) a control group, (ii) a low-dose ARB group, or (iii) a high-dose ARB group. The control group was fed tap water, while the low and high-dose ARB groups were fed commercially available losartan (Cozaar; Merck & Co, West Point,

Pennsylvania) dissolved in tap water at concentrations of 0.05 g/L, and 0.5 g/L, respectively. These doses were calculated based on the average fluid intake of a population of mice with similar demographic backgrounds to those tested in this study, and are well below reported toxicity levels. All animals were caged separately and allowed access to the water or ARB solutions ad libitum from the time of injury to the time of sacrifice. The animals were not pretreated in any way. The average daily fluid intake of each animal was monitored and recorded. Mice were sacrificed at 3 and 5 weeks. The injured muscles were isolated, mounted, and snap-frozen in liquid nitrogen-cooled 2-methylbutane. Samples were serially sectioned at 10 μm with a cryostat for histologic analysis.

Angiotensin Receptors in Skeletal Muscle

Immunohistochemistry was used to evaluate the presence and distribution of angiotensin II receptors in injured and uninjured skeletal muscle. Commercially available antibodies (Abcam Inc, Cambridge, Massachusetts) were used to localize angiotensin II receptors in skeletal muscle. The same sections were also stained with collagen IV antibodies (Biodesign International, Saco, Maine), which stain the extracellular matrix (basal lamina) of muscle fibers, and 4′-6-diamidino-2-phenylindole (DAPI) to delineate nuclear location. A blinded observer performed all analyses.

Muscle Fibrosis

To detect the amount of fibrosis in the area of injury, sections from each limb of each animal were washed in deionized water and stained with a Masson Modified IMEB Trichrome Stain Kit (IMEB Inc, San Marcos, California) according to the manufacturer's specifications. This technique, which stains nuclei black, muscle red, and collagen blue, was previously validated through immunohistochemistry as an accurate technique for evaluating fibrous tissue within skeletal muscle.[6,13,20] Five randomly selected 200× high-powered image fields within the injured area for each limb were obtained using a Nikon Eclipse 800 light microscope (Tokyo, Japan) fitted with a Spot-RT digital camera (Diagnostic Instruments, Sterling Heights, Michigan). Images were analyzed using Northern Eclipse image analysis software (Empix Imaging) to measure the percent area of collagen (blue staining tissue) within the injury zone. Color threshold levels within the software program were set to isolate the blue staining regions and calculate the area of that region that corresponded to the area of fibrosis. This value was expressed as a percentage of the entire cross-sectional area of the muscle section. A blinded observer performed all analyses.

Muscle Regeneration

Muscle sections were stained with hematoxylin and eosin. Muscle regeneration was assessed by counting the number of centronucleated myofibers. Five 200× high-powered fields throughout the injury zone were analyzed from each muscle, and the average number of regenerating myofibers per field was calculated. A blinded observer performed all analyses.

TABLE 1
Varying Concentrations of Angiotensin II With Myoblast (C2C12) and Fibroblast (3T3, PP2) Cell Lines Did Not Affect Proliferation at 24, 48, or 72 Hours[a]

Concentrations, M	24 h	48 h	72 h
C2C12			
0	125	114	160
10^{-8}	145	135	150
10^{-6}	130	140	200
10^{-4}	110	135	140
PP2			
0	25	25.5	40
10^{-8}	30	21.5	65.5
10^{-6}	25	24.5	37.5
10^{-4}	22	24.5	34.5
3T3			
0	102.5	570	1205
10^{-8}	111.5	611.5	1144
10^{-6}	124.25	612.5	1158
10^{-4}	141.875	139.8	112.7

[a]Angiotensin II concentration is expressed in molar amounts, and cell counts are expressed as thousands of cells ($\times 10^3$ cells).

Statistical Methods

Comparisons of muscle fibrotic area and number of regenerating myofibers data were performed by means of a 2-way analysis of variance (ANOVA) and Tukey's post-hoc test using SPSS software, version 16.0 (SPSS Inc, Chicago, Illinois). Immunohistochemical data are presented and compared qualitatively.

RESULTS

In Vitro

Angiotensin II does not affect cell proliferation. Angiotensin II appeared to have no effect on C2C12 (myoblast cell line), NIH 3T3 (fibroblastic cell line), or PP2 (fibroblast cell line) proliferation in vitro compared with controls at 24, 48, or 72 hours of culture (Table 1).

Angiotensin II increases the production of TGFβ-1 in fibroblasts. Fibroblasts isolated from normal skeletal muscle (PP2 cells) appear to up-regulate the expression of TGF-β1 detected by ELISA in the supernatant fluid when cultured with angiotensin II in a dose-independent manner at both 24 and 48 hours. National Institutes of Health 3T3 fibroblasts also appear to respond to the presence of angiotensin II by expressing TGF-β1, as detected by western blotting (data not shown).

In Vivo

Angiotensin II receptors in skeletal muscle. Immunohistochemistry demonstrated the presence of angiotensin II receptors in both normal and injured skeletal muscle. The

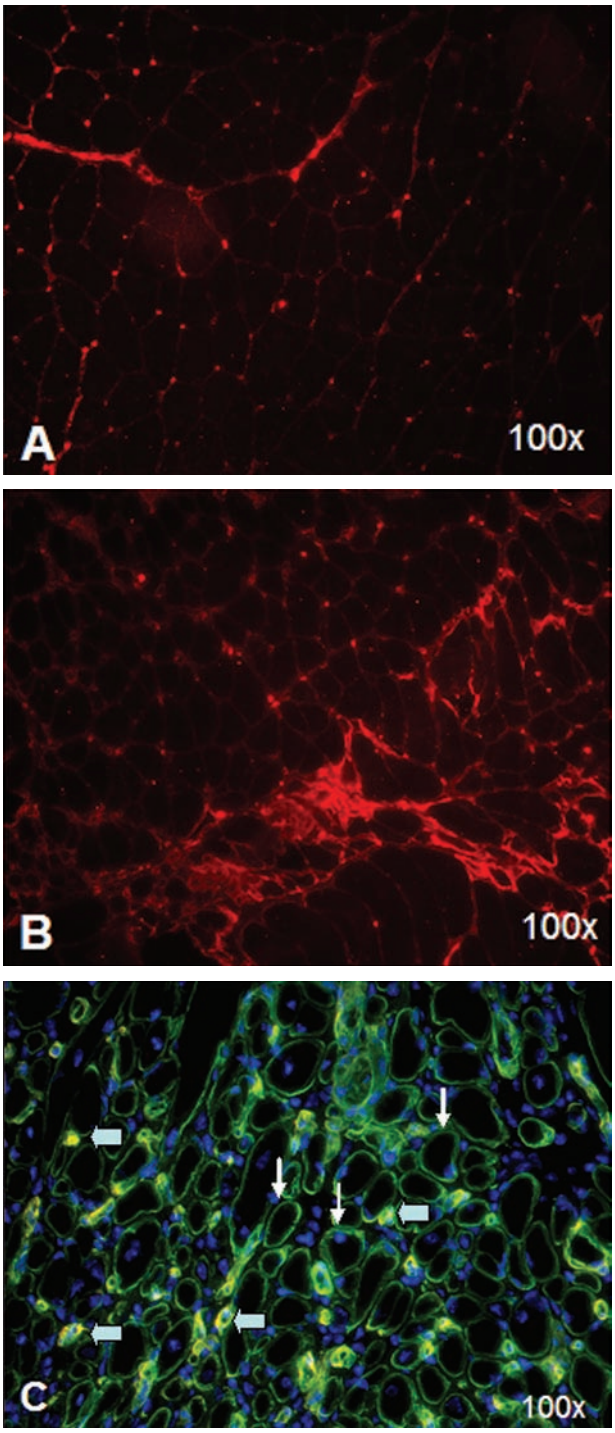

Figure 1. Immunohistochemistry demonstrating increased expression of the angiotensin II receptor (red) in injured (B) versus noninjured (A) skeletal muscle. C, the spatial relationships between collagen IV (green), angiotensin II receptor (red), nuclei (blue), and colocalization (yellow) are shown. The angiotensin II receptor distribution (thick arrows) appears more frequently within the extracellular matrix and sarcolemma of muscle fibers than within the myofibers themselves (thin arrows).

distribution of the receptors in normal muscle appeared to be sparse and evenly distributed along the cell membrane. After injury, the expression of angiotensin II receptors appeared elevated, particularly within the zone of injury. The expression of these receptors appeared to be greater within the cellular structure of the extracellular matrix, rather than on the membrane of the muscle fibers (Figure 1).

Animal Model

All mice used in this experiment were male. No mice demonstrated any ill effects from the treatment regimen, and there were no observable differences in the activity level in any of the groups; no deaths were attributable to the therapeutic intervention. The age of all animals at the beginning of the study ranged from 4 to 6 weeks, and the average weight was 21.4 ± 2.3 g. There was no significant difference in the weight of the animals in the experimental or control groups at any time point. The average dose of ARB received by each animal in the low-dose group and the high-dose group was approximately 0.027 ± 0.015 g/kg/d and 0.298 ± 0.20 g/kg/d, respectively.

Skeletal Muscle Regeneration

At 3 weeks, the average number of regenerating myofibers within the zone of injury (as identified by centrally located nuclei) was 56 ± 14 fibers per 200× high-powered field (n = 4) in the control group, 93 ± 25 (n = 8) in the low-dose group, and 95 ± 32 (n = 8) in the high-dose group (Figure 2). In injured animals treated for 5 weeks, a statistically significant increase in regenerating myofibers was observed. This increase was 45 ± 16 fibers per 200× high-powered field (n = 5) in the control group, 86 ± 19 (n = 8; $P < .01$ vs control group) in the low-dose group, and 103 ± 21 (n = 7; $P < .01$ vs control group) in the high-dose group. At both 3 and 5 weeks, the authors observed a trend toward increasingly greater numbers of regenerating myofibers as both the dose and duration of treatment increased (Figure 2).

Skeletal Muscle Fibrosis

Three weeks after muscle injury, without benefit of treatment, the control group had an area of fibrosis within the zone of injury equal to 34% ± 17% (n = 4). The low-dose treatment group had significantly less fibrosis within the zone of injury (16% ± 9%; n = 8; $P < .05$) when compared with the control group. The high-dose treatment group also had significantly less fibrosis (7% ± 4%; n = 8; $P < .01$ vs control) when compared with the control group. At 5 weeks after injury, the control group, low-dose group, and high-dose group had fibrosis areas of 45% ± 22% (n = 5), 19% ± 9% (n = 8; $P < .01$ vs control), and 14% ± 11% (n = 7; $P < .01$ vs control), respectively, demonstrating a similar trend toward an attenuation of fibrosis mediated by the administration of losartan (Figure 3).

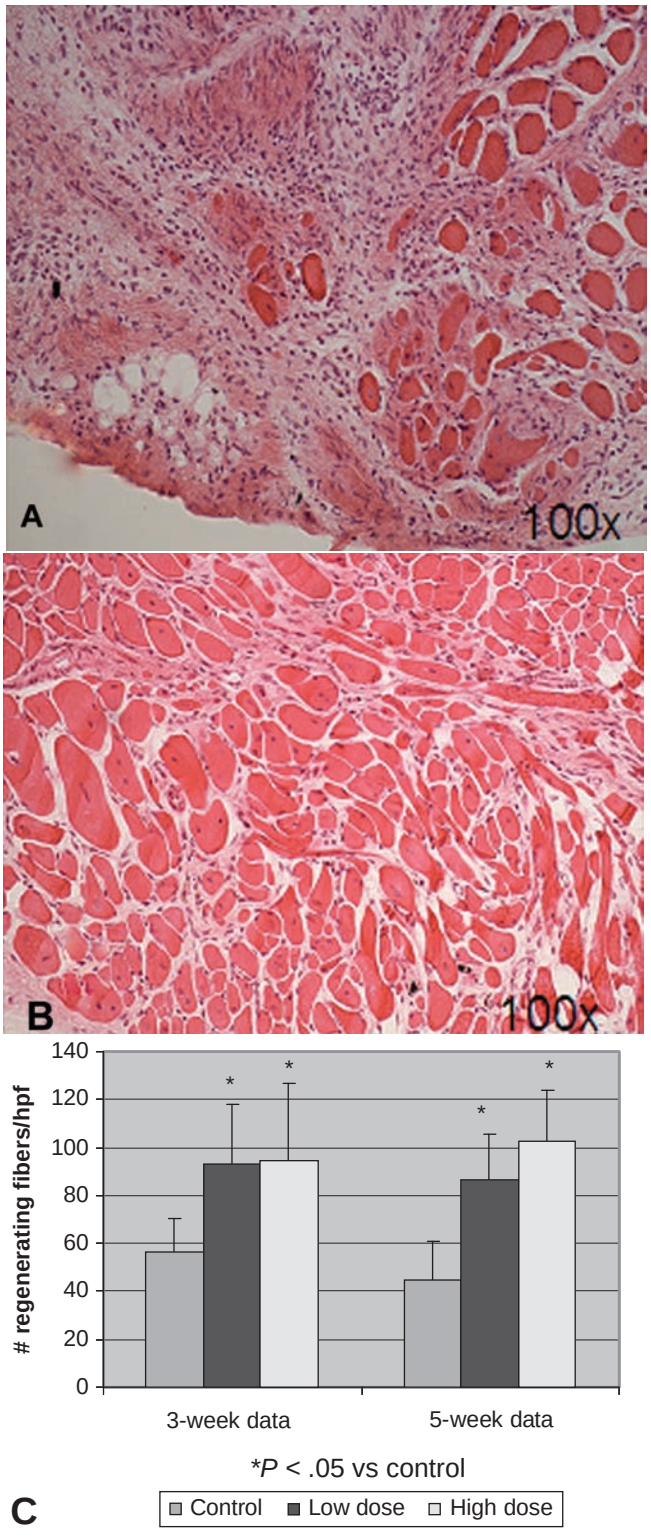

Figure 2. Characteristic hematoxylin-and-eosin–stained sections taken 5 weeks after laceration injury of the gastrocnemius muscle. Note the greater number of regenerating myofibers (recognized by their centrally located nuclei) in the high-dose angiotensin receptor blocker (ARB)–treated group (B) compared with the control group (A). The graph (C) depicts an increase in the number of regenerating myofibers in ARB-treated animals compared with controls. Error bars represent standard deviation. hpf, high-powered field.

DISCUSSION

The impetus for investigating the antifibrotic properties of ARBs in muscle healing arises from prior research on angiotensin II, a known vasoconstrictor and target of many antihypertensive medications, demonstrating its deleterious effects on smooth and myocardial muscle tissues after injurious insults. The role of angiotensin II in muscle fibrosis after injury is well documented in the cardiac literature, in which antagonism of angiotensin II with ARBs is noted to significantly improve cardiac contractility and cardiac output.[15] Moreover, histologic analysis indicates that the mechanism of cardiac improvement is related to decreased fibrosis and enhanced regeneration. The effect of ARBs on skeletal muscle healing, however, remains largely unstudied until recently.

The natural history of skeletal muscle injury is that of residual fibrosis within regenerate tissues that predisposes muscle to reinjury and reduced functionality, thereby constituting a significant clinical burden. After injury, the initial phase of myofiber regeneration wanes over time as it is replaced by fibrous tissue. A reduction of fibrous tissue and the improvement of muscle regeneration correlate with enhanced function.

Several investigations have observed this by treating injured skeletal muscle with antifibrotic agents that directly antagonize TGF-β1, and the digestion of existing fibrous tissue deposits appears to enhance myofiber regeneration.[5,9,13,14,23,24] Research on the pathogenesis of fibrosis further reveals that local levels of TGF-β1 during the fibrotic phase are elevated along the zone of injury[21] as a potential result of angiotensin II.[10]

In a mechanism consistent with these studies, we show through ELISA and western blot assays that fibroblasts increase TGF-β1 production when they are exposed to angiotensin II. We also show that angiotensin II receptors are more densely distributed within the extracellular matrix of the zone of injury, suggesting that angiotensin II receptor upregulation is intimately related to the deposition of fibrous tissue. Our results further demonstrate that inhibiting angiotensin II with an ARB diminishes post-traumatic fibrosis.

We have previously focused our attention on muscle injury models such as ischemia, contusion, strain, and cardiotoxin injection. While skeletal muscle laceration is a less frequently observed injury pattern, its histopathology is nearly identical to the more commonly observed strain injury patterns. Our purpose in choosing the laceration model in this experiment was 2-fold. First, it represents a reproducible model for evaluating skeletal muscle injury, which is a challenge with the more common strain-type injury model because of difficulty in accurately reproducing and delivering the same magnitude of injury between samples. Second, the laceration model establishes a "worst-case scenario" in which myofibers are completely transected. Observations of any treatment effect in this worst-case injury model would likely be of equal or, perhaps, greater benefit in less severe and more commonly encountered problems like strain injuries. In addition, the animals were not pretreated with ARB before injury, as this more closely resembles clinically relevant scenarios in which muscle injuries are treated. While many potential

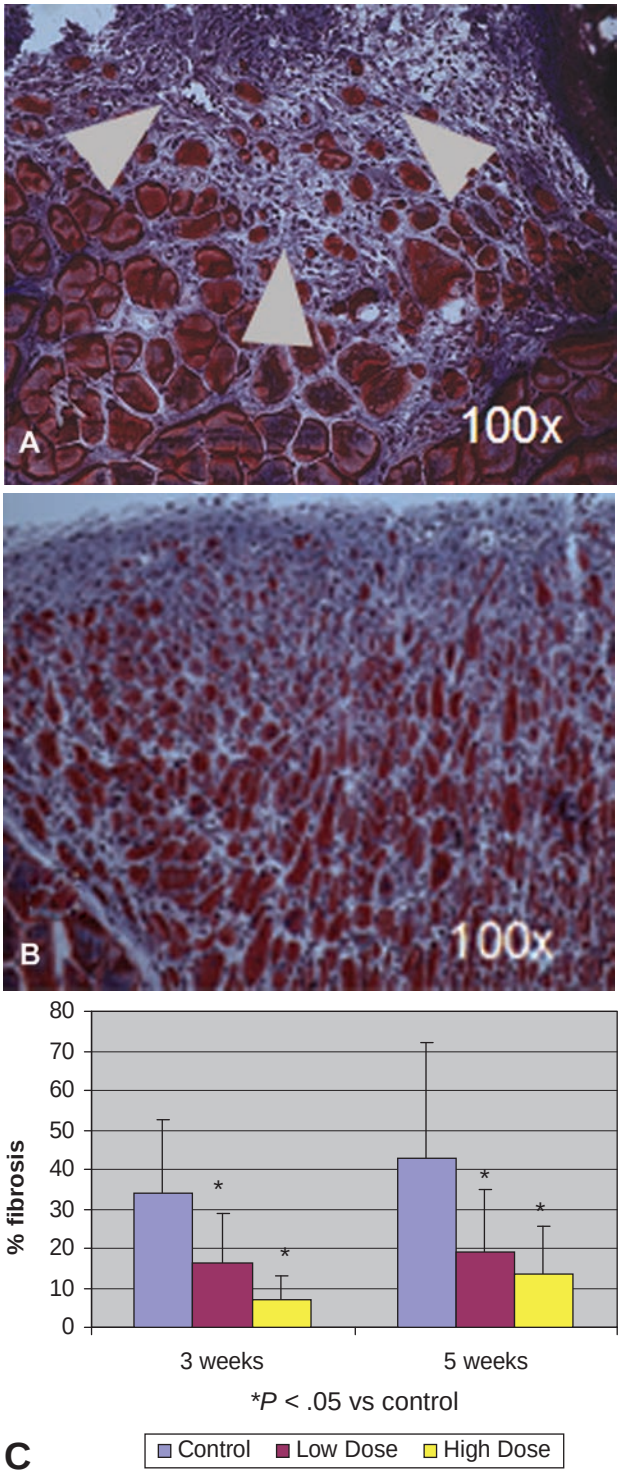

Figure 3. Masson's trichrome (collagen: blue, myofibers: red, nuclei: black) staining of sections taken 5 weeks after laceration injury of the gastrocnemius. Note the dense blue staining fibrous tissue in the control group (A) (arrowheads) compared with the high-dose angiotensin receptor blocker (ARB)–treated group (B). Both pictures are taken at 100× magnification. The graph (C) depicts a decrease in the amount of fibrosis in ARB-treated animals compared with controls. Error bars represent standard deviation.

problems exist with the use of an antihypertensive medication, all doses administered to the animals in this study were below the corresponding human dose equivalent for the antihypertensive effect of losartan. Accordingly, there were no ill effects observed in any of the animals.

Treating skeletal muscle-injured mice with an ARB for extended periods of time reduces the amount of fibrosis and enhances the number of regenerating myofibers in the zone of injury. This occurs in a duration-dependent manner, where medication administered for longer periods of time provided greater regenerative benefit. The observation of a positive regenerative effect after only 5 weeks of treatment, as opposed to other models where treatment was given for up to 9 months, is of notable significance. Prolonged treatment for acute skeletal muscle injuries would seem to be a less attractive and less pragmatic approach.

Similar success in reducing residual fibrosis and enhancing myofiber regeneration, as well as success in improving function, has been observed in animal models, albeit after treatment with potentially harmful compounds. As noted above, we have demonstrated that decorin, suramin, gamma-interferon, and relaxin can all decrease residual fibrosis, enhance myofiber regeneration, and improve function in animal models of skeletal injury.[9,13,14,22,24] However, these compounds are not commonly employed in clinical practice, and several of these are known to have relatively severe side effects that may preclude their use in the treatment of muscle injury.

While the clinical implications of identifying efficacious antifibrotic therapies are particularly important in the field of sports medicine, they apply to an even broader spectrum of orthopaedic patients. Athletes with muscle strains often struggle with lengthy recovery periods in rehabilitation that can limit their duration of play and predispose them to recurrent injuries that oftentimes are more severe.[26] For orthopaedic trauma patients, contusions and lacerations are markedly common, and for all patients undergoing elective and emergent procedures, many surgical incisions are essentially controlled lacerations of skeletal muscle. In each type of patient, the quality of regenerate skeletal muscle and the rate of recovery are, ultimately, compromised and delayed by fibrosis. For some patients, this can result in prolonged immobilization with greater risks for developing a deep venous thrombus or pneumonia. Because the clinical use of angiotensin receptor II inhibitors is not currently approved for orthopaedic surgery, clinical trials are necessary to further determine the effect of this class of medication on skeletal muscle healing, as well as on the functional outcomes, morbidity, and mortality associated with skeletal muscle injury and prolonged rehabilitation.

In light of the possibility that our work may pave the way for clinical trials on the effect of losartan as an antifibrotic therapy to promote optimal skeletal muscle healing, it is important to draw attention to potential adverse effects of angiotensin II receptor antagonists. The side effect profile of ARBs is surprisingly minimal compared with other antihypertensive medications; the only reported dose-related side effect is hypotension, while non-dose–related adverse outcomes include headaches, dizziness,

weakness, and fatigue.[3] This is particularly worth mentioning because these side effects can theoretically affect athletic performance on the field as well as patient performance in a rehabilitation program. Accordingly, clinicians and therapists must be vigilant in monitoring for such effects. While uncommon, there have also been reports of raised liver enzymes, cholestatic hepatitis, and pancreatitis with losartan.[7,8,23] Finally, while ARBs are more well known for their renoprotective effects, any diuretic generally carries the potential for acute renal insufficiency, which can be a particular concern that especially warrants mentioning in settings where patients may be dehydrated from athletic exertion or blood losses in postoperative and trauma settings.

In summary, the use of the ARB losartan, a commercially available, generally well-tolerated, safe, and widely used antihypertensive medication, has been demonstrated in this study to enhance muscle healing by reducing fibrosis and enhancing muscle regeneration in doses below those required for any antihypertensive effect. These findings may represent the most clinically appealing and potentially far-reaching antifibrotic therapy developed so far for otherwise healthy individuals who have sustained acute skeletal muscle injuries.

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ORIGINAL ARTICLE

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Interaction Between Macrophages, TGF-β1, and the COX-2 Pathway During the Inflammatory Phase of Skeletal Muscle Healing After Injury

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Inflammation, an important phase of skeletal muscle healing, largely involves macrophages, TGF-β1, and the COX-2 pathway. To improve our understanding of how these molecules interact during all phases of muscle healing, we examined their roles in muscle cells in vitro and in vivo. Initially, we found that depletion of macrophages in muscle tissue led to reduced muscle regeneration. Macrophages may influence healing by inducing the production of TGF-β1 and PGE_2 in different muscle cell types. We then found that the addition of TGF-β1 induced PGE_2 production in muscle cells, an effect probably mediated by COX-2 enzyme. It was also found that TGF-β1 enhanced macrophage infiltration in wild-type mice after muscle injury. However, this effect was not observed in COX-$2^{-/-}$ mice, suggesting that the effect of TGF-β1 on macrophage infiltration is mediated by the COX-2 pathway. Furthermore, we found that PGE_2 can inhibit the expression of TGF-β1. PGE_2 and TGF-β1 may be involved in a negative feedback loop balancing the level of fibrosis formation during skeletal muscle healing. In conclusion, our results suggest a complex regulatory mechanism of skeletal muscle healing. Macrophages, TGF-β1, and the COX-2 pathway products may regulate one another's levels and have profound influence on the whole muscle healing process.
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Tissue naturally responds to injury caused by trauma, microorganisms, toxins, or other causes through inflammation. Cytokines including histamine, bradykinin, serotonin, prostaglandins and others are released by damaged tissue and cause fluid leakage from blood vessels. These cytokines also attract white blood cells to infiltrate and migrate to the injury site to digest pathogens and dead/damaged cells, a process called phagocytosis. White blood cells and cytokines are of paramount importance for protection against infection and the invasion of foreign substances. Furthermore, they are involved with the regeneration of damaged tissues (Prisk and Huard, 2003; Tidball, 2005).

Skeletal muscle injury is a common type of injury associated with sports activities, high speed vehicle accidents, and military combat. In injured skeletal muscle, the inflammation response starts rapidly after trauma, and causes symptoms that include muscle pain, swelling, fever, and loss of mobility. To relieve these symptoms, many different non-steroidal anti-inflammatory drugs (NSAIDs) are used to block the cyclooxygenase (COX) enzymes and the products of the COX pathway, the prostaglandins. Early studies reported that NSAIDs had a favorable effect in quickly reducing muscle weakness and functional loss in the short term following muscle injury (Hasson et al., 1993; Obremsky et al., 1994; Mishra et al., 1995; Dudley et al., 1997). However, some studies reported either no effect or a detrimental effect on long term muscle strength recovery after prolonged NSAIDs treatment (Mishra et al., 1995; Almekinders, 1999). Recently, studies using COX-2 specific inhibitors also showed that the COX-2 pathway, which is mostly induced in pathological situations, is important in promoting muscle regeneration and reducing fibrosis formation (Bondesen et al., 2004; Shen et al., 2005). Furthermore, it is suggested that the prostaglandins, although the cause of uncomfortable symptoms, are actually beneficial in promoting skeletal muscle healing (Horsley and Pavlath, 2003; Pavlath and Horsley, 2003; Prisk and Huard, 2003).

Interestingly, it has been suggested that two other components of inflammation, macrophages and transforming growth factor-beta1 (TGF-β1), are related to the COX-2 pathway. Macrophages are an important source of COX-2 enzymes and prostaglandins during the inflammation phase

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(Graf et al., 1999; Lee et al., 2002). And, by some reports, the inhibition of the COX-2 pathway reduced the infiltration of macrophages to the injury site (Bondesen et al., 2004; Shen et al., 2005). TGF-β1, well known for its fibrotic effect, was also shown to be important in the inflammation phase as a result of its connection to the COX-2 pathway. TGF-β1 may increase the production of prostaglandin E_2 (PGE_2) through COX-2, and PGE_2 may inhibit fibroblast proliferation and collagen production to balance the fibrotic effect of TGF-β1 (Goldstein and Polgar, 1982; McAnulty et al., 1997; Keerthisingam et al., 2001). Furthermore, macrophages and TGF-β1 may also be connected to one another, as several studies have shown that macrophages may be an important source of TGF-β1 (Khalil et al., 1989, 1993; Wolff et al., 2004). However, little evidence of these interactions has been demonstrated in muscle cells during skeletal muscle injury.

To further the understanding of the healing mechanism of skeletal muscle injury and the role of inflammation in the muscle healing process, we examined the relationship between the COX-2 pathway, macrophages, and TGF-β1 in skeletal muscle inflammation. We found that these important components form a complex network wherein they appear to regulate each other. These interactions may not only modulate muscle inflammation, but also influence the regeneration and fibrosis phases of skeletal muscle healing.

Materials and Methods

Cell isolation and culturing

Myogenic precursor cells (MPCs) were isolated via a previously described preplate technique (Rando and Blau, 1994; Qu et al., 1998). Briefly, gastrocnemius muscles (GMs) were removed from 4-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), and minced with scissors. The minced tissues were enzymatically digested by sequential exposure to collagenase, dispase, and trypsin. The muscle cell extracts were then plated on collagen-coated flasks, and different populations were isolated by re-plating the extracts after different time intervals. The late plated population is usually composed of MPCs that have high myogenic potential when induced by low serum culture medium (Qu et al., 1998). Exudate macrophages were isolated according to the technique described before (Robertson et al., 1993). Briefly, mice were injected intraperitoneally with 30 ml thioglycollate broth (BD Biosciences, San Jose, CA). Three days later, the peritoneal macrophages were harvested by centrifugation. Macrophages and MPCs were used for in vitro experiments along with two other cell lines, NIH 3T3 fibroblasts and C2C12 myoblasts. These three types of cells were maintained in proliferation medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), and 0.5% chicken embryo extract).

ELISA assay

A low serum-containing medium (DMEM supplemented with 1% FBS and 1% HS) was used to culture cells in the experiments of growth factor and cytokine expression. The media from the TGF-β1 (5 ng/ml, Sigma, St. Louis, MO) and PGE_2 (0, 100, 1,000, or 10,000 ng/ml) treatments experiment were collected and kept at −80°C pending enzyme-linked immunosorbent assay (ELISA). The assay was performed as suggested by the manufacturer's protocols (DE0100 PGE_2 ELISA kit, DE1150 $PGF_{2\alpha}$ ELISA kit, R & D Systems, Inc., Minneapolis, MN).

Animal model

The Animal Research and Care Committee at the authors' institution approved all experimental protocols for this study (Protocol 29/04). Twenty-four C57BL/6J mice (males, 6 weeks of age, Jackson Laboratories), 4 COX-2 knock-out mice (males, 6 weeks of age, Taconic farm), and their wild-type controls (males, 6 weeks of age, Taconic farm) were used for in vivo experiments. The GMs of the mice were injected with cardiotoxin (CTX, c3987, Sigma), a snake venom, to create a muscle injury. Briefly, the mice were anesthetized by intraperitoneal injection of 0.03 ml ketamine (100 mg/ml) and 0.02 ml xylazine (20 mg/ml). Ten microliters of diluted CTX (or 9μl CTX + 1μl TGF-β1) was injected in the middle mass of each GM. The mice were sacrificed at different time points after injection (1, 3, 5, or 14 days). The GMs were harvested from both legs for either flow cytometry or histological analysis. For the latter purpose, the GMs were fresh-frozen in 2-methylbutane pre-cooled by liquid nitrogen, and stored at −80°C pending cryosection.

Macrophage depletion

Clodronate liposomes, were prepared as described previously (Seiler et al., 1997; Tyner et al., 2005). The liposomes act as carriers for clodronate, which is toxic to phagocytic cells. Two days before muscle injury, 1 mg of clodronate liposomes (20 mg/ml) was injected intraperitoneally into C57/BL 6J mice to deplete macrophages. Mice injected with empty liposomes were used as controls. Flow cytometry using macrophage marker antibodies (F4/80 and CD-11b) was used to verify the extent of macrophage depletion.

Hematoxylin and eosin (H & E) staining

The cryosections were fixed in 1% glutaraldehyde for 1 min, and then immersed in hematoxylin for 30 sec. After washing with alcohol acid and ammonia water, they were immersed in eosin for 15 sec. After each step, sections were rinsed with distilled water. The sections were then dehydrated by treatment with alcohols of increasing concentrations (70%, 80%, 95%, and 100%). Following this, the sections were treated with xylene and covered with glass slips. Slides were analyzed on a bright field microscope (NIKON Eclipse E800, Nikon, Tokyo, Japan). Northern Eclipse software (Empix Imaging, Cheektowaga, NY) was used to measure the minor axis diameters of centronucleated regenerating myofibers (i.e., the smallest diameter of a myofiber across the central nucleus; 200× magnification; 200 random myofibers obtained from four samples/group).

Western blot

After washing with PBS, Laemmli sample buffer (BioRad, 161-0737, Hercules, CA) was applied to the surface of culture dishes to collect proteins from live cells. For muscle tissue samples, serial cryosections of 10 μm thickness were collected in Eppendorf tubes and treated with T-PER tissue protein extraction agent (78510, Pierce, Rockford, IL) and mixed with Laemmli sample buffer (BioRad, 161-0737). Protein samples were boiled for 5 min, separated on 10% SDS–polyacrylamide electrophoresis gels, and transferred to nitrocellulose membranes. Mouse anti-COX-2 (160112, Cayman, Ann Arbor, MI) and anti-TGF-β1 antibodies (555052, BD Biosciences Pharmingen, San Diego, CA) were applied as primary antibodies, and mouse anti-β-actin (Sigma; 1:8,000) was used for protein quantification. The horseradish peroxidase-conjugated secondary antibodies (Pierce) were diluted to 1:5,000 (v/v). Blots were developed by using SuperSignal West Pico Chemiluminescent substrate (Pierce), and positive bands were visualized on X-ray film. All results were analyzed by Northern Eclipse software (Empix Imaging).

Flow cytometry

The GMs from non-TGF-β1-treated (10 μl cardiotoxin injection) and TGF-β1-treated groups (9 μl cardiotoxin plus 1 μl of 5 ng/ml TGF-β1 injection) were surgically removed before injury, and at 1, 2, 3, and 5 days after injury for serial evaluation. Collagenase, dispase, and trypsin were used to digest the tissue matrix and

isolate the cells. Debris was removed via filtration with 70 μm filters.

Cells were treated with 10% mouse serum (Sigma) to block non-specific binding sites. Primary rat anti-CD-11b (conjugated with FITC, R & D Systems, Inc.) and rat anti-F4/80 (conjugated with APC, BD Biosciences, San Jose, CA) antibodies were used in combination to identify neutrophil and macrophage populations. We added 7-amino-actinomycin D (7-AAD; BD Biosciences Pharmingen, San Diego, CA) to exclude non-viable cells from the analysis. Samples were then analyzed via fluorescence activated cell sorting (FACS) Caliber flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences).

Statistics

A Chi-squared analysis was used to analyze the percent differences between the number of macrophages identified by flow cytometry and western blotting results. Comparisons between two groups were made by the use of an unpaired Student's *t*-test. All other data were analyzed by one-way ANOVA statistical analysis. Post-hoc multiple comparison tests were performed to determine which means differed. A value of $P < 0.05$ was considered statistically significant. Error bars on figures represent the standard deviation.

Results

Macrophage depletion and muscle regeneration

Liposome clodronate was injected intraperitoneally 2 days prior to muscle injury to deplete the macrophage populations in mice. We used flow cytometry to quantify the number of infiltrating macrophages after injury. This macrophage infiltration peaked 2 days post-injury, and decreased quickly after 5 days. Liposome clodronate injection significantly reduced the number of infiltrating macrophages after muscle injury at all time points observed when compared to the empty liposome control group. At 1d, 2d, 3d, and 5d post-injury, liposome clodronate injection decreased macrophage infiltration by 73.2%, 80.2%, 77.4%, and 64.2%, respectively ($P < 0.05$, Fig. 1A). As shown on the flow cytometry graph, the macrophage population, which is shown in the S2 quadrant in the empty liposome control mice, was significantly reduced in the clodronate liposome-injected mice (Fig. 1B).

Clodronate liposome-treated mice exhibited reduced muscle regeneration at both 14 and 28 days after injury. The size of regenerating myofibers in liposome clodronate-treated mice was significantly smaller than those observed in the empty liposome control group ($P < 0.05$, Fig. 1C–E). However, at 7 days post-injury, there was no significant difference between these two groups.

Macrophages depletion decreases the expression of TGF-β1

Injured GMs were isolated from empty liposome control and clodronate liposome-injected mice. We examined the TGF-β1 expression level in injured muscle tissue by using Western blot analysis. Our results indicated that, compared to the empty liposome control group, TGF-β1 expression was significantly decreased 3 and 5 days after injury in the clodronate liposome-injected group, which had its macrophages depleted before the creation of muscle injury ($P < 0.05$, Fig. 2A,B). These results suggest that macrophages play an important role in inducing the expression of TGF-β1 during the inflammatory phase of skeletal muscle after injury.

TGF-β1 induces the production of COX-2 enzyme and prostaglandins

To examine the effect of TGF-β1 on the COX-2 pathway, we cultured MPCs and treated them with TGF-β1. We analyzed the COX-2 enzyme level from MPCs treated with TGF-β1 and the non-treated control cells for 4 days. Western blot analysis showed that COX-2 production was significantly higher in the MPCs treated by TGF-β1 compared to non-treated control cells ($P < 0.05$, Fig. 3A,B). We further tested the expression of PGE_2 from different muscle cells, including MPCs, NIH 3T3 fibroblasts, and C2C12 myoblasts, after they were treated with TGF-β1 for 4 days. We found that TGF-β1 treatment increased the expression of PGE_2 significantly in all cell types, when compared to non-treated control cells ($P < 0.05$, Fig. 3C).

To further validate the relationship between TGF-β1 and the COX-2 pathway, we used wild-type MPCs as a model system. In this experiment, NS-398 (a COX-2 specific inhibitor) was added to the cell culture to block the COX-2 enzyme activity. This additional treatment ablated the increased PGE_2 expression that was induced by adding TGF-β1 into the cell culture ($P < 0.05$, Fig. 3D). In addition, COX-$2^{-/-}$ cells had very low levels of PGE_2 expression, even if TGF-β1 was added. This finding suggests that TGF-β1 regulates the production of COX-2 pathway products, and that the enhanced expression of PGE_2 may be mediated by COX-2.

TGF-β1 increases the infiltration of macrophages through the COX-2 pathway

To examine the effect of TGF-β1 on macrophage infiltration, we created CTX injury (with or without TGF-β1 treatment) on the GMs of wild-type mice. We found that, after TGF-β1 treatment, the number of infiltrating macrophages was significantly increased compared with the CTX-only group at days 1, 2, and 3 after muscle injury ($P < 0.05$, Fig. 4A). The same experiment was conducted on COX-$2^{-/-}$ mice. Interestingly, we found that the number of infiltrating macrophages actually decreased with the addition of TGF-β1, instead of being increased 3 days after injury ($P < 0.05$, Fig. 4B). This further validates the suggestion that the effect of TGF-β1 on macrophage infiltration is mediated at least in part by the COX-2 pathway products.

PGE_2 decreased the expression of TGF-β1

To examine the influence of PGE_2 on fibrosis formation, we chose to examine the effect of PGE_2 treatment on the expression of TGF-β1, which is known for its fibrotic effect especially in skeletal muscle (Li et al., 2004). Three different muscle cell types, MPCs, NIH 3T3 fibroblasts, and C2C12 myoblasts, were tested for TGF-β1 expression following treatment with PGE_2 for 4 days. We found that PGE_2 treatment decreased the expression of TGF-β1 significantly in all cell types tested, when compared to non-treated control cells ($P < 0.05$, Fig. 5). This result suggests that PGE_2 may be able to decrease fibrosis formation after muscle injury by decreasing the expression of fibrotic growth factor TGF-β1.

Discussion

Inflammation is an important phase of skeletal muscle healing. Macrophages, TGF-β1, and the COX-2 pathway are the integral components of this phase. In order to further the understanding of muscle healing and improve the health care of muscle injury patients, we must investigate the importance of these components and elucidate their interaction in muscle healing regulation. In this study, we examined their roles and relationships during the inflammation phase and postulated a possible regulatory mechanism (Fig. 6).

Many researchers suggest that macrophages are important for muscle regeneration (Cantini et al., 2002; Camargo et al., 2003; Chazaud et al., 2003; Tidball, 2005). To create a "Loss of Macrophages Function" condition in skeletal muscle healing, especially in the inflammation phase, we used the clodronate liposome injection technique (Seiler et al., 1997; Tyner et al.,

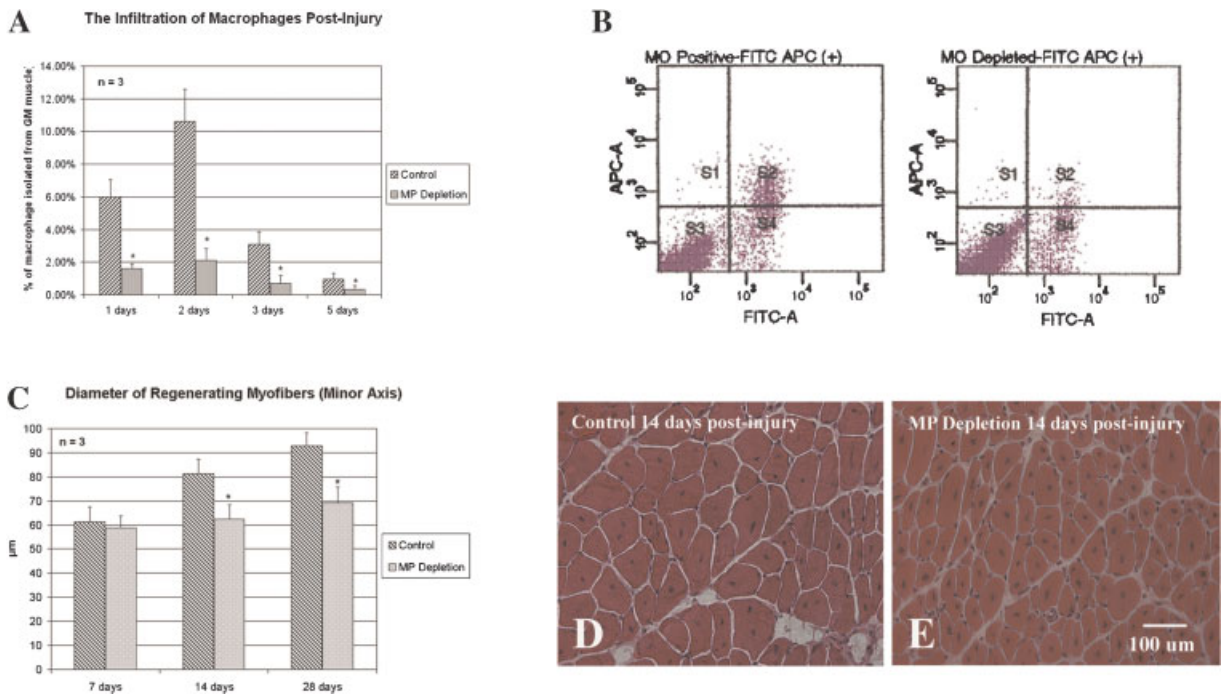

Fig. 1. Injection of clodronate liposome depleted macrophages in injured GM significantly after injury. At 1d, 2d, 3d, and 5d post-injury, liposome clodronate injection decreased macrophage infiltration by 73.2%, 80.2%, 77.4%, and 64.2%, respectively ($P < 0.05$, A). Flow cytometry results showed that the macrophage population, which was shown in the S2 quadrant in non-treated mice at 48 h after injury (B, left), was significantly reduced in clodronate liposome-treated mice (B, right). The X-axis represents the CD-11b cell surface marker; and the Y-axis represents the F4/80 cell surface marker. Compared with empty liposome-control group, clodronate liposome-injected mice exhibited significantly reduced sizes of regenerating myofibers 14 and 28 days post-injury ($P < 0.05$, C). Sample H & E stained GM sections from empty liposome and clodronate liposome-injected groups 14 days post-injury are shown in (D,E). The asterisks indicate a statistically significant difference ($P < 0.05$) between the marked groups and the control group. The error bars in the graph represent the standard deviation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2005) to deplete the macrophage populations in mice. With the injection of clodronate liposomes, most of the macrophages were depleted and only a few remaining cells infiltrated into muscle tissue during the inflammation phase. We found that, although regeneration of injured muscle did occur, the macrophage depletion group had significantly smaller myofibers than the non-treated group, which is indicative of a delay in muscle regeneration. This suggests that macrophages participate in the muscle healing process, and may play a beneficial role in the growth of regenerating myofibers.

One of the possible mechanisms by which macrophages contribute to the healing process is by inducing the proliferation and differentiation of satellite cells by secreting growth factors and cytokines (Cantini et al., 1994, 1995; Lescaudron et al., 1999; Merly et al., 1999; Chazaud et al., 2003). The factors secreted by macrophages exert their effects not only on specialized satellite cells during muscle regeneration, but have a broader mitotic activity on all myogenic cells (Cantini and Carraro, 1995). In this study, we found that macrophage-depleted mice had significantly lower TGF-β1 levels in their injured GM muscles compared to those from non-treated control mice. This finding suggests that macrophages play a role in modulating the level of these growth factors including TGF-β1 during the healing process of skeletal muscle. However, the exact role of macrophages and their interaction with other muscle cells warrants further investigation. Studies need to be performed to determine the cellular source of growth factors in the muscle inflammation process, and whether direct contact or humeral regulation is necessary to induce the expression of these growth factors.

Fibrosis is a complex biological process that is usually seen in severe muscle injury. Fibroblasts are activated to proliferate and produce abnormal amounts of extracellular matrix (ECM). Damaged skeletal muscle tissue is replaced by the deposition of overproduced ECM (i.e., fibrosis/scar) tissue, instead of regenerating myofibers. TGF-β1 is one of the most potent fibrotic stimuli (Li et al., 2004). It is an inducer of ECM protein synthesis and fibroblast proliferation (Kahari et al., 1991; Taipale et al., 1994), and it has been implicated in the fibrogenesis of various tissues (Border and Noble, 1994). However, the role of TGF-β1 in skeletal muscle healing is not limited to fibrosis. Previous studies have indicated that TGF-β1 may be an immunosuppressive molecule because its elimination or its downstream Smads signaling cascade disruption leads to severe inflammatory disease (Kulkarni and Karlsson, 1997; Yang et al., 1999; Nakao et al., 2000; Monteleone et al., 2004a). On the other hand, it has been suggested by some authors that TGF-β1 is able to increase PGE_2 expression in other tissues (Fawthrop et al., 1997; McAnulty et al., 1997; Keerthisingam et al., 2001; Han et al., 2004). Since it has been suggested that the COX-2 pathway products including PGE_2 and $PGF_{2\alpha}$ are important inflammatory mediators that induce regeneration in skeletal muscle healing (Bondesen et al., 2004; Shen et al., 2005), it is important to explore their relationship with TGF-β1. In our in vitro studies, we found that the addition of TGF-β1 significantly increased the production/expression of both COX-2 enzyme and PGE_2 in muscle cells. By blocking COX-2, TGF-β1's effect on PGE_2 expression was ablated. Furthermore, the effect of TGF-β1 was not observed in COX-$2^{-/-}$ cells. These results indicate that TGF-β1 is not only a fibrotic inducer,

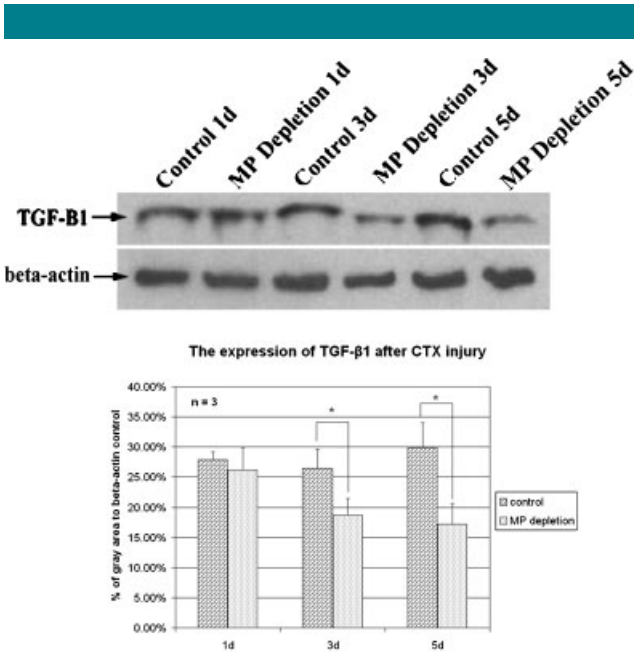

Fig. 2. The in vivo expression level of TGF-β1 was significantly lower in the clodronate liposome-injected mice compared to the empty liposome control mice 3 and 5 days after injury ($P < 0.05$). A representative western blot result is shown at the top. The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

but is also an inflammatory modulator in muscle injury. In fact, TGF-β1 may enhance the inflammatory response by enhancing the COX-2 pathway, especially the production of PGE_2. This may represent an important finding because it indicates that TGF-β1 may both up- and downregulate inflammation through different pathways.

It has been shown that TGF-β1 interferes with the inflammation phase in various ways (Fiocchi, 2001; Warshamana et al., 2002; Monteleone et al., 2004a,b; Wang et al., 2005). Mostly, TGF-β1 was thought to inhibit inflammation because TGF-β1 is a negative regulator of NF-κB activation (Haller et al., 2003). Smad7 maintains high NF-κB activity during inflammation by blocking TGF-β1 signaling (Monteleone et al., 2004a,b; Wang et al., 2005). To elucidate whether TGF-β1 modulates inflammation by other means, macrophage infiltration for example, we injected TGF-β1 together with CTX. The TGF-β1 addition significantly increased the infiltration of macrophages into injured skeletal muscle. This suggests that TGF-β1 interferes with the inflammation phase of muscle healing by increasing the number of infiltrating macrophages. However, in COX-$2^{-/-}$ mice, the number of infiltrating macrophages was decreased with the addition of TGF-β1 to the cardiotoxin injection. This finding suggests that the COX-2 pathway mediates this TGF-β1-induced macrophage infiltration. As prostaglandins are known to cause localized vasodilation, it is reasonable to postulate that they may be at least partly responsible for the chemotaxis of macrophages. In future experiments, prostaglandin's chemotactic property as well as TGF-β1's regulation of NF-κB should be more deeply investigated. If it could be shown that TGF-β1 also has a negative impact on inflammation through the downregulation of NF-κB, it would be intriguing to discover which pathway is dominant in muscle injury, and determine why TGF-β1 would have contradictory effects. Based on the findings that macrophages increased the expression of TGF-β1, and TGF-β1 increased the infiltration of macrophages, which is mediated by the COX-2 pathway, they may form a positive feedback loop to further enhance the number of infiltrating macrophages in the injured muscle (Fig. 6).

A previous study of liver fibrosis showed that PGE_2 inhibited TGF-β1-mediated induction of collagen alpha I production in

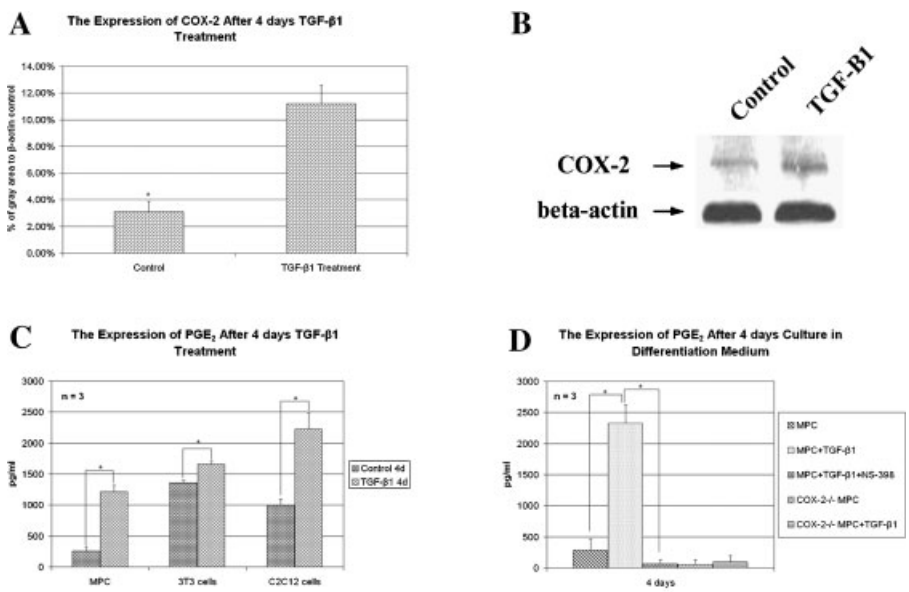

Fig. 3. MPCs that were treated with TGF-β1 for 4 days had significantly higher production of COX-2 enzyme than the non-treated control cells ($P < 0.05$, A). A representative Western blot result is shown in (B). Muscle cells that were treated by TGF-β1 for 4 days had significantly higher expression levels of PGE_2 compared with the non-treated control cells ($P < 0.05$, C). NS-398 can significantly reduce the increased PGE_2 expression that was induced by TGF-β1 ($P < 0.05$, D). COX-$2^{-/-}$ MPC cells, both treated and non-treated with TGF-β1, expressed PGE_2 at very low levels. The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

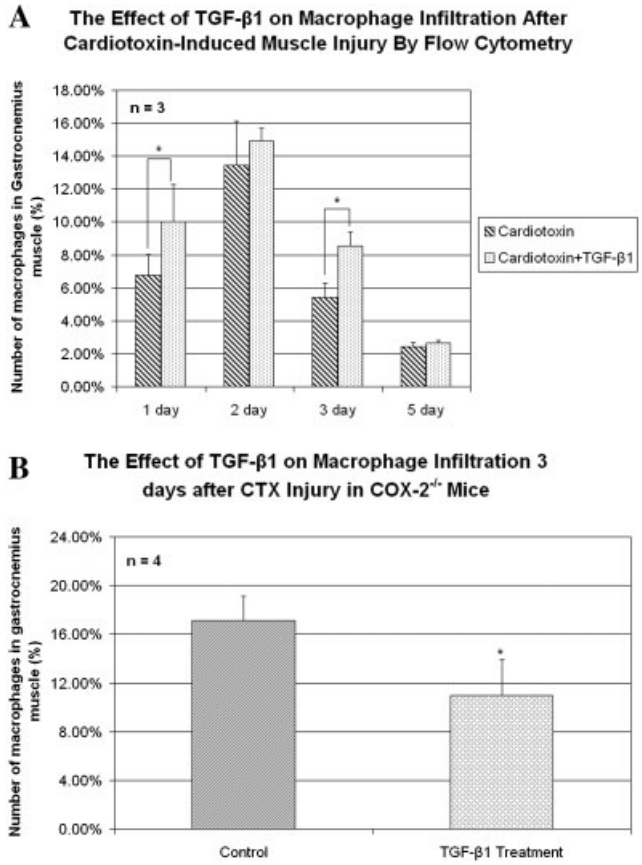

Fig. 4. Flow cytometry results showed that CTX injection can create injury in skeletal muscle and induce the infiltration of macrophages. The addition of TGF-β1 significantly increased the number of infiltrating macrophages at 1 and 3 days after CTX-induced muscle injury ($P < 0.05$, A). In contrast, CTX-injured COX-2$^{-/-}$ mice showed significantly decreased numbers of infiltrating macrophages 3 days post-injury ($P < 0.05$, B). The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

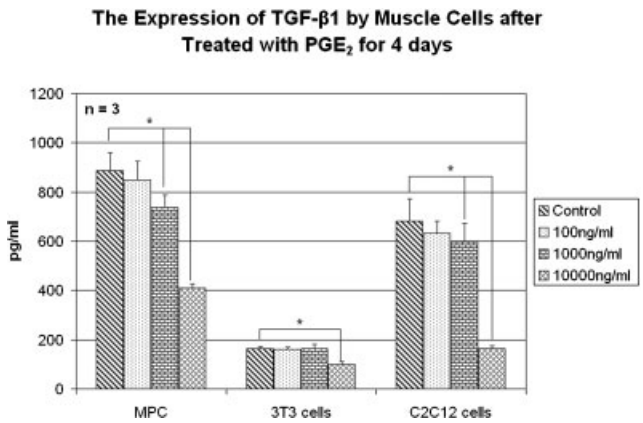

Fig. 5. At concentrations of 1,000 ng/ml and 10,000 ng/ml, PGE_2 decreased the expression of TGF-β1 significantly in both MPCs and C2C12 myoblasts. However, PGE_2 was able to decrease the expression of TGF-β1 in NIH 3T3 fibroblasts only at a concentration of 10,000 ng/ml ($P < 0.05$). The asterisks indicate a significant difference ($P < 0.05$) between the compared groups. The error bars in the graph represent the standard deviation.

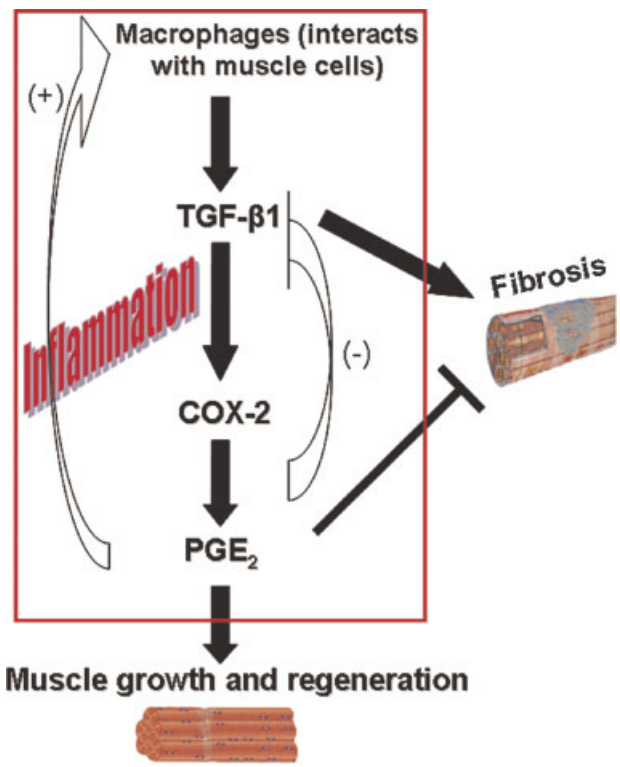

Fig. 6. The proposed mechanism of muscle inflammation is shown. Macrophages interact with muscle cells to increase the level of TGF-β1 in muscle inflammation. TGF-β1 increases the level of prostaglandins, which mediates muscle regeneration and the further infiltration of macrophages, forming a positive feedback loop. In turn, PGE_2 decreases the expression of TGF-β1 and forms a negative feedback loop to downregulate the fibrosis formation. Macrophages, muscle cells, TGF-β1, and COX-2 pathway form a complex network to regulate each other, and thus modulate the following regeneration and fibrosis formation during the healing process after injury. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

hepatic cells (Hui et al., 2004). This finding suggests that PGE_2 and TGF-β1 are able to regulate one another's levels by forming a negative feedback loop resulting in homeostasis of fibrosis formation. To test this phenomenon in skeletal muscle, we treated different muscle cell types with PGE_2 and analyzed their TGF-β1 expression. What we found was that PGE_2 treatment decreased the expression of TGF-β1 significantly in all muscle cell types, when compared to non-treated control cells. On the other hand, our previous study showed that by using NS-398 to block COX-2, and thus the expression of PGE_2, the expression of TGF-β1 was increased in injured muscle tissue (Shen et al., 2005). These results indicate that the TGF-β1 level was probably regulated by PGE_2. The application of NS-398 probably inhibited PGE_2 expression and led to a high level of TGF-β1, and subsequent fibrosis formation in injured muscle. Our findings are consistent with the results from a previous liver fibrosis study (Hui et al., 2004), and further suggests the existence of a negative feedback loop between TGF-β1 and PGE_2 in skeletal muscle (Fig. 6).

PGE_2 is also a potent inhibitor of fibroblast proliferation (Goldstein and Polgar, 1982; Durant et al., 1989) and collagen

synthesis (Goldstein and Polgar, 1982; Saltzman et al., 1982). This suggests that PGE_2 may play an important role in minimizing ECM production. This is especially important in an environment favoring fibrosis formation, such as is found within the inflammation of damaged liver. Interestingly, our preliminary data showed that different concentrations of PGE_2 may affect the proliferation of muscle cells differently (data not shown). Thus, the next step in our study would be to assess the level of PGE_2 in injured skeletal muscle, which should give further insight into the effect of PGE_2 on muscle cell proliferation. It is also important to investigate the role of other prostaglandins, such as $PGF_{2\alpha}$, PGE_2, and 15-PG dehydrogenase in the future, because other prostaglandins may also play a role in the muscle healing process, and 15-PG dehydrogenase may have a significant effect on prostaglandin levels.

In summary, our study demonstrates that the COX-2 pathway, macrophages, and TGF-β1 are important components of the inflammation phase of injured skeletal muscle. Inflammation affects the overall healing of skeletal muscle through these cellular and molecular components. In addition, we found that these components may modulate the production of each other and form a complex co-regulatory mechanism. As we begin to comprehend the fact that simply blocking inflammation by using NSAIDs has a potential downside on muscle healing, more studies are warranted to improve the quality of healthcare in patients afflicted with skeletal muscle injuries.

Acknowledgments

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Blocking Myostatin by AAV2-Delivered Myostatin Propeptide Improves Muscle Cell Transplantation

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INTRODUCTION

Myostatin (MSTN) is a potent negative regulator of muscle growth [1] and we have found that like TGF-β1, MSTN also contributes to the formation of fibrosis in injured skeletal muscle [2]. JacqueTremblay's group has successfully blocked MSTN signaling in *mdx* mice by generating 2 types of transgenic *mdx* mice. The 1st carries a dominant negative form of the MSTN receptor (dnActRIIB) and the 2nd over-expresses follistatin (FSTN), which is an inhibitor of MSTN. Normal myoblasts transplanted into these transgenic *mdx* mice outperformed myoblasts transplanted into nontransgenic *mdx* mice [3, 4]; nevertheless, dnActRIIB and FLST do not specifically inhibit MSTN but also interfere with other growth factors such as activins. In the current study we utilized an adeno-associated viral vector carrying the MSTN propeptide gene (AAV-MPRO) to specifically inhibit the action of MSTN. MPRO directly binds the MSTN molecule and has been shown in previous studies. We have proved that blocking MSTN with MPRO both increase muscle regeneration and reduce the formation of fibrosis at the site of injury 4 weeks following injury (unpublished data). In the current study we injected AAV-MPRO/GPF into the skeletal muscles of *mdx*/SCID mice 4 weeks prior to muscle progenitor cell transplantation to investigate whether blocking MSTN signaling in host muscle by MPRO can elevate the regeneration capacity of donor muscle cells in dystrophic muscle. The success of cell therapy for treating muscle injuries and diseases has been limited by the poor survival and function of the donor cells after transplantation; therefore, improving the microenvironment in the host dystrophic muscle prior to cell transplantation is an alternative approach to enhance the efficiency of cell transplantation.

METHODS

Animal model: All experiments in this study were approved by the Children's Hospital of Pittsburgh IACUC. AAV2-MPRO ($1x10^{11}$ v.g.) in 50 µl of PBS was injected into the gastrocnemius muscles (GMs) of 3 *mdx*/SCID mice (4 weeks of age); the same amount of AAV-GFP was injected into the GMs of mdx/SCID littermates as controls. Four weeks after injection of AAV, 300,000 muscle progenitor cells (MPCs) were injected into each of the GMs of AAV treated *mdx*/SCID mice (n = 6), and the mice were sacrificed 2 weeks following the cell transplantation. Masson's Trichrome staining was performed to identify fibrous scar tissue in the mice. Northern Eclipse software (Empix Imaging, Inc.) was used to measure the diameters of regenerating myofibers within the injection site and also measure the areas of fibrous scar tissue. Dystrophin staining was performed and the dystrophin-positive myofibers were then counted to assess the efficiency of cell transplantation in the skeletal muscle of the *mdx*/SCID mice. Student's *t* test was used to determine significance ($P<0.05$).

Immunohistochemistry: Muscles were snap frozen in liquid nitrogen, cyrosectioned and the tissue sections were then fixed in 5 % formalin for 5 minutes, followed by 3 washes with PBS. The sections were blocked with 10% donkey serum for 1 hour, and incubated overnight at 4°C in either a goat MPRO antibody (2.5 ug/ml, RnD system) or a rabbit dystrophin primary antibody (1:500, Abcam) that was diluted in 5% donkey serum. The following day, the sections were washed with PBS and incubated with the corresponding secondary antibodies, rabbit anti-Goat or donkey anti-rabbit IgG conjugated with Alexa594.

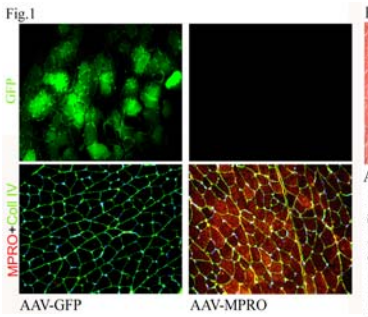
RESULTS

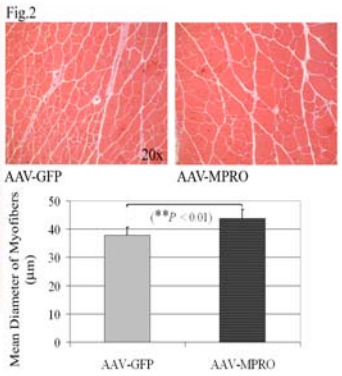
Gene expression in muscle: Expressions of GFP (green) and MPRO (red) in AAV-GFP/MPRO transduced muscles indicates that genes delivered by AAV were stably expressed in skeletal muscle (Fig. 1).

Accelerated muscle regeneration in MPRO over-expressing mdx/SCID mice: Six weeks after AAV2-MPRO transduction, a significant increase in muscle weight was observed compared to the GFP transduced control (data not shown). Moreover, the mean diameter of the muscle fibers in the AAV-MPRO transduced muscles was significantly larger than that of AAV-GFP transduced muscles (Fig. 2).

Reduced fibrosis: Masson's trichrome stain showed a reduction of fibrous scar tissue (blue) in the AAV2-MPRO transduced GMs as compared to the AAV-GFP control muscles (Fig. 3).

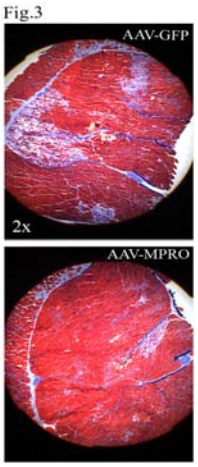
Improved cell transplantation in the GMs of AAV-MPRO transduced mdx/SCID mice: Fig.4 shows the representative images of dystrophin-positive muscle fibers in the muscles of *mdx*/SCID mice 2 weeks after cell transplantation. Normal MPCs injected into AAV-MPRO transduced dystrophic muscle surpassed the regeneration capacity of cells injected into AAV-GFP transduced control muscle. This was determined by counting the total number of dystrophin positive myofibers (Fig. 4)

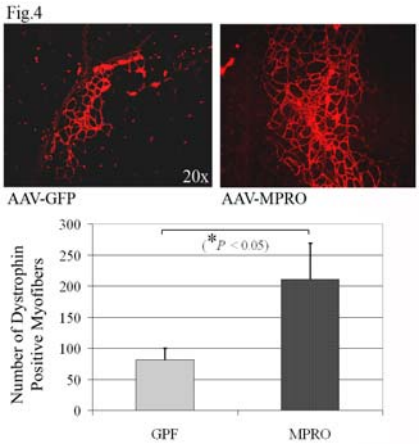




DISCUSSION

Our results revealed stable expression of AAV-mediated GFP and MPRO in transduced skeletal muscle. MPRO ameliorated the dystrophic pathology of *mdx* mice by promoting muscle regeneration and reducing collagen deposition. Inactivation of MSTN in dystrophic host muscle by MPRO significantly improved the success of MPC transplantation as compared to the AAV-GFP transduced control, evidenced by significantly more dystrophin-positive myofibers in the former. Two mechanisms may account for this improved cell transplantation. First, MPRO counteracts the inhibitory effect that MSTN has on donor cell regeneration. Deregulating the suppression of MSTN on muscle cells by MPRO transduction may augment donor cell proliferation, differentiation, and hence increase dystrophin-positive myofiber formation. Second, the improved cell transplantation in *mdx* mice by MPRO may be partially accredited to the fact that MPRO inhibits MSTN, thereby reducing fibrosis formation in dystrophic muscle. The ameliorated milieu in host dystrophic muscle may favor donor cell survival and engraftment. Taken together, the combination of gene therapy and cell therapy has been shown to be a potential effective and novel approach for treating injured and diseased muscle.





ACKNOWLEDGMENTS

This work was supported by funding from the Henry J. Mankin Endowed Chair for Orthopaedic Research at the University of Pittsburgh, the William F. and Jean W. Donaldson Chair at Children's Hospital of Pittsburgh, the Hirtzel Foundation, and the National Institutes of Health (R01 AR47973 awarded to J.H.).

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Angiotensin II Receptor Blocker Ameliorates Skeletal Muscle Healing in a Dose Dependent Manner

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INTRODUCTION:

Muscle injuries are very common musculoskeletal problems encountered in sports medicine. Although these injuries are capable of healing, complete functional recovery is hindered by the formation of dense scar tissue triggered by TGF-β1 [1]. We have previously reported that several agents such as decorin and suramin can inhibit fibrosis and improve regeneration in injured skeletal muscle. The safety of these agents, however, remains unknowns to use those agents for treating muscle injury. By contrast, Losartan (LOS), one of the Angiotensin II Receptor Blockers (ARBs)—is a FDA approved antihypertensive medication and has been shown to also be antifibrotic in a variety of tissues, including skeletal muscle [2]. This ARB has a well-tolerated side-effect profile, can also block TGF-β1 to attenuate the development of pathological fibrosis. In this study, we investigated optimum dose of LOS in treating injured muscle to help the translation of this research from bench to bedside.

METHODS:

Cell proliferation assay: C2C12 myoblasts and fibroblasts obtained from murine skeletal muscle by using the previously published preplating technique [3], were cultured in 96 well plates (n=5) with DMEM containing 10% FBS,1% penicillin/streptomycin (P/S), with addition of different concentration of either Angiotensin II (ANG) (10^{-12} to 10^{-4} M) or LOS (10^{-12} to 10^{-4} M). CellTiter Cell Proliferation Assay kit (Promega, Madison, WI) was used to measure cell proliferation at 24, 48, and 74 hour after incubation.

C2C12 cell differentiation assay: C2C12 myoblasts were cultured in 24 well plates (n=4) with differentiation medium (DMEM supplemented 2% horse serum and 1% P/S) containing different concentration of ANG (10^{-12} to 10^{-4} M) or LOS (10^{-12} to 10^{-4} M). Three days after incubation immunocytochemistry of myosin heavy chain was performed and the fusion index (a ratio of the number of nuclei in myotubes/total nuclei) was calculated to assess the effects of ANG and LOS on differentiation capacity of C2C12 myoblast.

Animal model: The muscle contusion model was developed in tibialis anterior (TA) muscle of C57BL/6 wild-type mice. Different concentrations of LOS (12.5, 125, 1250, or 4000mg) in 1Liter of tap water were administered beginning immediately after injury until endpoint while the control group drink tap water (six mice in each group). These doses were calculated based on the average fluid intake of mice as 3, 30, 300, and 1000mg/kg/day respectively. Animals were sacrificed and TA muscles were harvested 4 weeks after injury. Muscle samples were then cryosectioned and histological stained (hematoxlin and eosin stain (H&E) and Masson's Trichrome stain). The numbers of centronucleated regenerating myofibers were counted to evaluate the regeneration.Northern Eclipse software (Empix Image, Inc.) was used to quantitate the total cross-sectional area of fibrosis. Statistical analysis was performed with ANOVA and Scheffe's F test as post hoc test. Statistical significance was defined as $p < 0.05$.

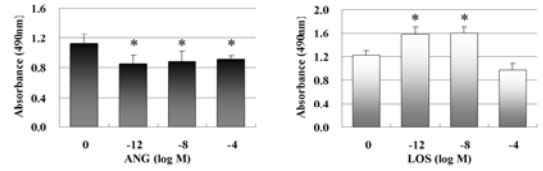
RESULTS SECTION:

LOS enhanceed C2C12 myoblast proliferation: Exposure to 10^{-12} to 10^{-4} M of ANG attenuated C2C12 proliferation after 72hr incubation. Whereas, 10^{-12} to 10^{-8} M of LOS stimulated C2C12 proliferation at the same time point. However, a high concentration of LOS (10^{-4} M) failed to increase cell growth (Fig. 1). Surprisingly, neither ANG nor LOS had any effect on fibroblast proliferation (not shown).

LOS stimulated myoblast differentiation: C2C12 differentiation was inhibited by 10^{-12}M of ANG, however, the effect attenuated at higher concentrations (Fig.2). Likewise, 10^{-12} M of LOS enhanced C2C12 differentiation and the stimulation was dropping while concentrations of LOS increased. It is noteworthy that 10^{-4} M of LOS significantly suppressed differentiation (Fig. 2).

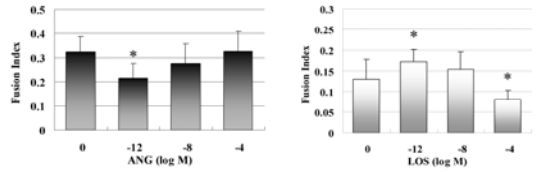
LOS enhanced muscle regeneration and decreased fibrosis: We could observe significant increases in number of centronucleated myofibers in the LOS-treated animals when compared with control animals (Fig. 3). The group treated with 300 mg/kg/day of LOS showed the most effective regeneration among groups. The best regeneration correlated with the group that displayed a significant reduction in fibrosis. However, these beneficial effects decreased when the dose of LOS was increased to 1000 mg/kg/day (Fig. 4).

Figure. 1



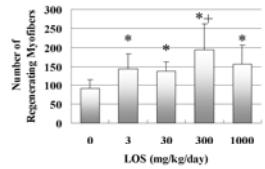
*: $p < 0.05$ vs control

Figure. 2



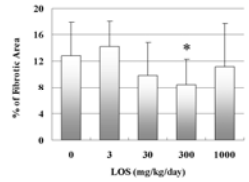
*: $p < 0.05$, vs control

Figure. 3



*: $p < 0.05$, vs control
+: $p < 0.05$, vs other trreated group

Figure. 4



*: $p < 0.05$, vs control

DISCUSSION:

The biphasic effect of LOS on C2C12 myoblasts *in vitro*, stimulating at low dose while decreasing at high dose, suggested that there is an optimal dose of LOS. Consequently we found that LOS improves skeletal muscle regeneration at 4 weeks after contusion injury, except that these effects were reduce/eliminated in 1000 mg/kg/day group. The best effective dose was 300 mg/kg/day. Overall, these effects of LOS were more pronounced for regeneration than for fibrosis. These *in vivo* results are consistent with our *in vitro* results that LOS was able to exert effects on C2C12 whereas fibroblasts were not affected by either ANG or LOS. Regeneration and fibrosis are two competitive processes after muscle injury; therefore, decreasing fibrosis observed in LOS-treated group is can be the result of the increase regeneration. In other words, LOS might indirectly reduced fibrosis by directly stimulating regeneration. Although there were not statistical differences in fibrosis in lower dose LOS groups (3 and 30 mg/kg/day) as compared to control, these effects of LOS on both regeneration and fibrosis showed similar dose dependent trend. However, *in vivo* results above were obtained only from single time point after injury. Since there are time lags between peaks of myofiber regeneration and fibrosis after injury, further investigations are required to examine the effect of LOS on regeneration and fibrosis at their individual peak time, which will facilitate clinical application of ARBs in improving skeletal muscle healing.

ACKNOWLEDGEMENTS:

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Blocking Myostatin Improves Muscle Healing Via Enhancement of Angiogenesis

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INTRODUCTION

Myostatin (MSTN), a member of the TGF-β superfamily, is a powerful inhibitor of skeletal muscle growth [1], and has also been implicated in the formation of fibrosis in injured skeletal muscle [2]. An increase of muscle regeneration and a decrease of fibrosis is observed in the injured muscle of MSTN knockout (MSTN-/-) mice when compared to that of injured wild-type (WT) mice [2]. We have also observed similar results when we block MSTN utilizing adeno-associated viral vector to deliver MSTN propeptide (AAV-MPRO) (unpublished data), a natural inhibitor of MSTN [3]. In this study, we investigated whether AAV-MPRO could stably express in vivo and benefit the healing of injured skeletal muscle up to one year after muscle injury. We further examined mechanisms by which the inhibition of MSTN improved skeletal muscle healing after laceration injury. A positive correlation has been reported between angiogenesis and skeletal muscle healing [4]; therefore, we hypothesized that injured MSTN-/- muscles and injured muscles transduced with AAV-MPRO would show more angiogenesis than their respective controls.

METHODS

Animal experiments: We injected AAV-MPRO ($1x10^{11}$ v.g.) in 50 µl of PBS into gastrocnemius muscles (GMs) of C57BL/6 WT mice (6-8 weeks old). AAV-GFP was injected into GMs of WT littermates as a control. One month after AAV vector transduction, the GMs of the mice were lacerated. The GMs of 10 mice were harvested at 4 weeks post-injury (n = 5). The remaining mice were sacrifice at 1 year post-injury (n = 4). HE staining was performed to monitor muscle regeneration in the injured muscles. All samples were stored in -80°C.

Immunohistochemistry: The muscles were cryosectioned and fixed in formalin. The sections were then incubated with 10% HS for 1 hour after which a rat CD31 (endothelial marker) primary antibody (1:150) was applied along with a mouse anti-fast myosin heavy chain (MyHC) antibody or sheep anti-MPRO antibody, which were incubated for 1 hour at RT. The sections were then washed three times with PBS and incubated with the secondary antibodies rabbit anti-rat conjugated with 555 (red) and anti-mouse conjugated with 488 (green) or anti-sheep-555 (red) for 1h. Nuclei were counter stained with DAPI (blue). We used image J software to measure the number of CD31-positive capillaries to evaluate angiogenesis within the injury site. Student's *t* test was used to determine significance ($P<0.05$).

RESULTS

Expression of GFP and MPRO in skeletal muscle: One year after AAV transduction, we still detected strong expressions of GFP and MPRO in myofibers of AAV-GFP/MPRO transduced muscles (Fig. 1). MPRO-positive myofibers (arrowheads) indicates constitutive expression of MPRO in skeletal muscle transduced with AAV-MPRO.

Improved skeletal muscle healing by MPRO: We observed significantly larger regenerating myofibers in AAV-MPRO transduced injured muscle than in controls, 1 year post-injury (Fig. 2A, C). Correspondingly, distribution of diameter of regenerating myofibers revealed that AAV-MPRO transduced injured muscle contained a higher percentage of larger diameter regenerating myofibers. For instance, 30% of the diameters of regenerating myofibers in control muscle were larger than 50 µm when compared to 61 % of that in AAV-MPRO transduced muscle (Fig. 1B). Blocking MSTN with MPRO led to a significant increase in the weight of the GMs (Fig. 2D).

Increased angiogenesis in AAV-MPRO-transduced mice after GM laceration: Fig.3 shows representative images from injured AAV-MPRO transduced muscles and controls. Significantly more CD31-positive capillaries were observed in AAV-MPRO transduced injured muscle than in controls at 4 weeks post-injury (Fig.3).

DISCUSSION

Our unpublished data show that angiogenesis occurs earlier in the MSTN-/- muscle than in normal muscles after laceration injury. At 4 weeks after injury, MSTN-/- muscle contained significantly more CD31-positive capillaries than the controls. The increased angiogenesis appears to partially account for improved muscle healing in injured MSTN-/- mice. Nevertheless, it is noteworthy that the irreversibility of genetic transfer may result in compensatory upregulation, developmental defects, etc. We therefore used AAV to deliver MPRO cDNA into the GMs of WT mice in order to restrict MSTN propeptide over-expression in the certain skeletal muscles of adult mice. With this model, we injected AAV-MPRO particles into skeletal muscle prior to creating a laceration injury in the mice. Our results suggest that AAV-MPRO could be stably expressed in vivo and could improve the healing of the injured muscle, long term. We found that MPRO gene transfer improved skeletal muscle healing with increased muscle regeneration. Muscle transduced with MPRO also showed a greater amount of angiogenesis than untransduced controls which coincided with the increased regeneration and decreased fibrosis 4 weeks after injury (data not shown). Taken together, this data suggests a negative correlation between MSTN and angiogenesis in injured skeletal muscle; however, the clear relationship between MSTN and angiogenesis warrants further investigation.

Fig.1

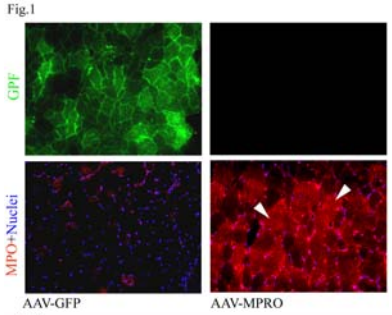


Fig.2

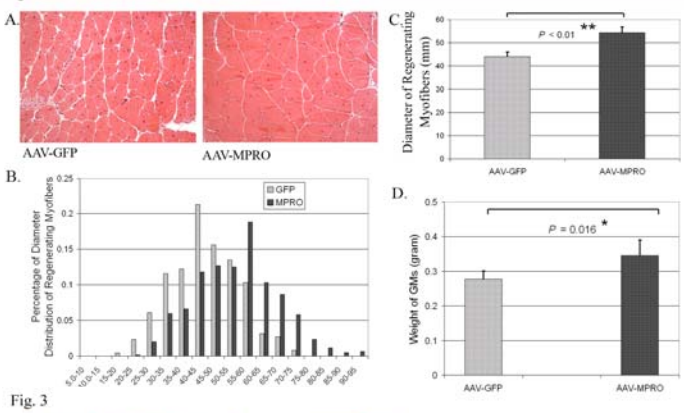
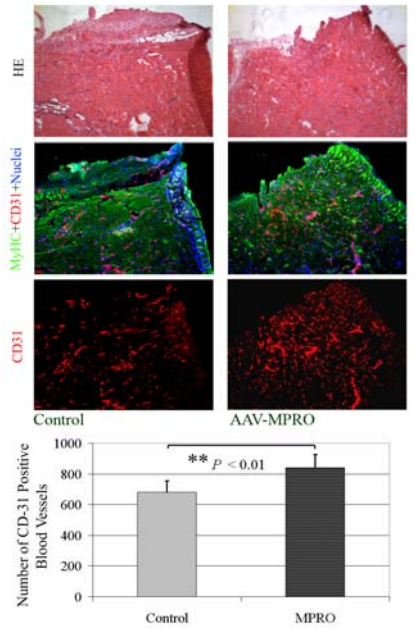


Fig. 3



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Improving Recovery after a Recurrent Hamstring Injury with an Angiotensin II Receptor Blocker Therapy: a Case Report and a Review of Literature

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Introduction

Skeletal muscle fibrosis is a major limiting factor in the complete functional recovery of injured skeletal muscle. Our laboratory has investigated several anti-fibrotic agents for minimizing the pathologic fibrotic cascade after injury including, decorin, suramin, gamma interferon (IFN-γ), relaxin and an angiotensin II receptor blocker (ARB) losartan to antagonize transforming growth factor (TGF-β1). Inhibition of TGF-β1, which is a key player identified in the fibrosis pathway, is the most promising in animal studies. Losartan is particularly well-suited for the clinical use as it is already approved by the Food and Drug Administration (FDA) for indications such as hypertension and congestive heart failure, with an extremely safe side-effect profile. Moreover, losartan is not a prohibited substance in sports, which would be an important consideration for athletes and sports medicine professionals who care for the injured athlete.

Hamstring muscle injuries are common in both athletic and non-athletic populations with a recurrence rate cited up to 34%[1]. Time to recovery and prevention of recurrent injuries impact not only quality of life, but also ability to return to sport and return to work in many instances. Improving recovery from these injuries could thus have great clinical and even financial implications. Currently typical treatment for hamstring injuries includes rest and immobilization immediately following injury and then a gradually increasing program of stretching, strengthening, and return to activity [2]. Despite the considerable medical need, there has been relatively little progress in the development of therapeutic approaches to enhance healing following this injury.

In this case study, we describe a case of a collegiate football player whose treatment for a recurrent hamstring strain was safely augmented with losartan. The

subject was informed that data concerning the case would be submitted for publication, and he consented.

Case Report

History of Present Illness

The subject was a 21 year-old collegiate football punter. He presented 10 days after an acute onset of "searing" pain in his left posterior thigh pain when he was kicking with his left leg planted. He had experienced a less severe, but similar injury 5 weeks prior (midseason) that was diagnosed as a mild mid-hamstring strain and was treated with relative rest and rehabilitation. The symptoms for the initial injury resolved within a week. After the recurrent injury, the patient experienced increased pain and was unable to play in the last game of the season. Treatment prior to the clinic visit included rest and 5 days of ibuprofen only.

Physical Examination

On initial physical examination 10 days after the injury, the subject walked without an antalgic gait. He had no obvious deformity but exhibited a small area of superficial ecchymosis over the distal hamstring without any tenderness. The proximal biceps femoris was tender to palpation at about 3 cm inferior to the left gluteal fold, without a palpable defect. The subject exhibited a popliteal angle of 30 degrees (30 degrees deficit in knee extension with the hip flexed to 90 degrees) on the injured side compared to 5 degrees on the uninjured side. His average injured hamstring strength

measurements with a hand-held dynamometer at 30 and 90 degrees of flexions were 47% and 54% of the uninjured side respectively. An MRI was obtained on the same day and demonstrated a partial thickness tear of the biceps femoris at the proximal myotendinous junction with surrounding edema without an associated avulsion fracture or hematoma. This was consistent with the diagnosis of an acute Grade 2 hamstring strain (Fig.2).

Treatment and Outcome

After obtaining the subject's informed consent for treatment, he was started on a 30-day treatment course of losartan at the manufacturer's recommended dose of 50 mg per day by mouth. The subject underwent a routine rehabilitation supervised by his athletic trainer along with the pharmaceutical treatment, gradually progressing to eccentric strengthening. He was followed every 7-10 days with serial measurement of hamstring flexibility and strength (Figure 1). The subject reported no side effects while he was taking the study medication and remained normotensive throughout.

By the third week after the injury, no deficit was evident in hamstring flexibility. By the ninth week, the subject reported essentially no pain at rest or with normal activity, and he had only minimal pain when the hamstring muscle was under tension. Isometric hamstring strength measurements at this point at 30 and 90 degrees of flexion were 92 and 84% of the uninjured side respectively (Fig.1).

Eleven weeks after the injury, an isokinetic test of the hamstrings was performed showing an essentially normal result. Peak hamstring torque, on the left injured side, was 96% of the uninjured side at 60 degrees per second and 107% of the uninjured side at 180 degrees per second. There was a slight decrease in the work during the maximum

repetition for the hamstrings at 60 degrees per second (12.9%) and at 180 degrees per second (9%). In terms of total work performed, the injured side (over 5 repetitions at 60 degrees per second) showed there was a 10.7% deficit compared to the uninjured side. At 180 degrees per second, there was a 9.8% deficit in total work across the 10 repetitions. He did exhibit a bit more fatigue of the hamstrings on the injured side at 180 degrees per second. Comparing the work in the 1st 1/3 of the repetitions to the last 1/3 of the repetitions, the decrease in hamstring torque on the injured side was 19% compared to 8.3% on the uninjured side. Finally, bilaterally the subject's hamstrings were weak relative to the quadriceps when tested at 60 degrees per second (hamstring to quadriceps ratio was 49.6% on the injured side and 51.2% on the uninjured side – this should have been on the order of 60 to 65%). In summary, all isokinetic testing parameters were within a normal range compared to the contralateral leg.

A second MRI study was also obtained at that time of isokinetic testing which revealed a thickened proximal hamstring tendon with minimal residual edema compared to the uninjured side (Fig.3).

Discussion

Muscle injuries are common in recreational and professional athletes alike, constituting 10% and 55% of all injuries sustained by athletes, depending on the type of sport [3-14]. While relatively minor muscle injuries, such as strains, can heal completely without intervention, severe muscle injuries typically result in the formation of dense scar tissue that impairs muscle function and can lead to muscle contracture and chronic pain. Scar formation at the site of injury has also been cited as a cause for early recurrent injury

due to the reduced tensile strength of the scar. Collagen type III, which is a major component of the immature healing scar is easy to fail with unsuitable loading forces [1].

The hamstring muscle complex is the most commonly injured [15-17] and occurs in sports requiring sprinting, bursts of speed or rapid acceleration [18]. A recent study on National Football League training camp injuries during the past decade reports hamstring injuries were the most common muscle strain as well as the most severe, with an average of 8.3 days lost per event [19]. Recurrence is common secondary to the pressure to return to play prior to complete healing of the injury with a higher re-injury rate than any other type of injury [2]. One study cited the rate of recurrent hamstring injury was 17% in runners and 12% in football players [20]. Powell and Zarins reported 16% recurrence rate in the recovery timeframe (2002 NFLTP meeting).

Recurrent injuries can lead to decreased performance as well as additional lost time from competition. Recovery from a re-injury takes longer to rehabilitate than the initial injury (12 days vs. 9 days) (Powell and Zarins, 1989-1998, 1696 injuries in NFL players as reported at 2002 NFLTP meeting) and in extreme cases, a recurrent hamstring injury can be a season, or career-ending injury. In a study of 546 professional rugby players, an average of 25 days were lost from competition secondary to recurrent injuries compared to 14 days for acute strains [21].

While current evidence for the optimal management of hamstring strain injury is inconclusive, increased understanding of the cellular and molecular events that occur during fibrosis of various tissues has provided a strong foundation for the development of anti-fibrotic therapy and improved muscle healing. TGF-β1 plays a crucial role in tissue

fibrosis [11–23], particularly in skeletal muscle [9, 10, 24–27] and warrants attention as a key target for antifibrotic applications.

In an effort to test various methods of antagonizing the effect of TGF-β1, we have investigated the abilities of decorin, suramin, IFN-γ, relaxin and an angiotensin II receptor blocker to inactivate TGF-β1. Animal studies in our lab have shown that blocking TGF-β1 can decrease fibrosis and improve muscle healing [10, 35-41]. Unfortunately, many of the antifibrotic agents that have been investigated in the laboratory setting cannot readily be translated to clinical practice due to lack of an oral formation, FDA-approval status, and relatively severe side-effect profile.

Losartan, an angiotensin II receptor blocker does not share these issues and is an attractive alternative. Losartan is currently used widely for treatment of hypertension and congestive heart failure, and is commercially available in a daily oral formulation. In addition, losartan is not a banned substance for use in athletic populations. Losartan was the first orally active, non-peptide angiotensin receptor antagonist described [22] and has been extensively studied.

The renin-angiotensin system is believed to play a role in the initiation and progression of fibrosis via collagen synthesis in several organs [23], including the heart, lung, kidney, liver, as well as skeletal muscle [24-37]. Of particular interest for the purpose of this case study, in the skeletal muscle system losartan has shown to normalize muscle architecture, repair and function in an animal model of Marfan syndrome [38]. ACE inhibitors have also been shown to improve muscle mass in older patients [39].

Losartan has been used in normotensive subjects and had little side effects. Goldberg evaluated tolerability and blood pressure effects of losartan in a multiple-dose

study in 14 healthy male subjects.[40] The authors found good tolerability and a modest blood pressure-lowering effect. Adverse clinical effects were mild and included hypotension, headache, dizziness, weakness and fatigue. Rare side effects including elevated liver enzymes, cholestatic hepatitis, and pancreatitis have also been reported. Among these, only hypotension and acute renal insufficiency would be of relevance in most athletic populations, especially when athletes consume nonsteroidal anti-inflammatory drugs (NSAIDs) concomitantly which inhibit free water clearance and cause acute renal failure.

The optimal dose of losartan for antifibrotic effect in humans has not yet been determined, because there is no precise, established method to translate the experimental dose of losartan used in animals to humans. The manufacturer's recommended starting dose of losartan for antihypertensive and congestive heart failure therapy is 50 mg per day, and clinical doses range from 25 mg to 100 mg per day. The pilot study of 14 subjects by Sookoian for hepatic fibrosis had employed the conventional starting dose of 50 mg per day which showed benefit of losartan [33]. The subject of this case study tolerated the 50 mg per day dose of losartan well and did not report any side effects while he was on the medication.

Our previously published laboratory animal experiments have demonstrated the up-regulation of angiotensin II receptors in posttraumatic skeletal muscle tissue [41]. In our *in vitro* study, fibroblasts within the extracellular matrix in the zone of injury expressed an increased level of TGF-β1 when they were exposed to angiotensin II. In follow-up *in vivo* studies, losartan effectively decreased fibrosis and improved skeletal muscle healing in regenerating muscle tissue in a dose-dependent manner after laceration injury [41]. This result was duplicated in animal studies with another muscle injury model, utilizing

contusion, to examine the effects of different doses of losartan four weeks after injury. The mean area of fibrosis of the control group within the zone of injury was 12.80 +/- 4.98%. Higher doses of losartan (30 and 300 mg/kg/day) had significantly smaller mean areas of fibrosis (9.82 +/- 4.93 and 8.48 +/- 3.82%, respectively) when compared with control group ($p<0.05$). Lower doses of losartan (3 and 10 mg/kg/day) did not affect scar tissue formation after contusion injury.

In situ physiological tests to evaluate the muscle strength showed that the control group (contusion injury without losartan therapy) had significantly less specific peak twitch and tetanic force (P-value=0.001 and 0.025, respectively) when compared with the non-injured group. The group treated with 30mg/kg/day of losartan had increased peak twitch and peak tetanic force indicating greater muscle strength when compared to control. The minimum effective doses for specific peak twitch and maximum force were 30 and 10mg/kg/day group, respectively. The timing of administration of losartan to block the fibrosis cascade also influences the muscle healing. The different doses of losartan were started after injury at 0, 3, 7 and 14 days after injury and were maintained for four weeks. When losartan administration was started 3 or 7 days after the injury, a significant improvement in specific muscle twitch was seen.

Although the recovery course of hamstring injuries is quite variable from one athlete to another, available historical data on the outcomes for a majority of acute hamstring injuries indicates that pain and stiffness during activities of daily living resolve within 1 to 2 weeks. Edema lasts 6 to 10 weeks and injuries most commonly involve biceps femoris at the proximal musculotendinous junction.

Several studies have determined the time to return to play after hamstring injuries (Table 1). Pomeranz had retrospectively studied 14 professional athletes and identified several poor prognostic factors for prolonged rehabilitation time greater than 6 weeks, including complete tendinous or myotendinous junction rupture, hyperintensity or hemorrhagelike signal intensity on T1-weighted images, large cross-sectional area involvement (>50%), distal tendinous or myotendinous lesions, and localized ganglionlike peritendinous fluid collections. [42]

A study by Slavotinek et al. included at 37 players from 3 Australian Rules football teams with acute hamstring injuries.[43] In contrast to the previous study by Pomeranz, there was no correlation between the location of the injury and rehabilitation time, but an abnormal cross-sectional muscle area greater than 50% was associated with longer rehabilitation time. In this study, days lost from competition ranged from 13 to 48 days with median of 27 days.

On the other hand, Connell reported the best prognostic indicator for MRI was the longitudinal length of the injury from his study of 60 professional Australian football players.[44] Other associated factors were injury to the biceps femoris muscle, cross-sectional injury area, and injury outside of the musculotendinous junction. In this study, the days to return to play ranged between 4 to 56 days with a median of 21 days.

Another study of 546 players from professional rugby union in United Kingdom reported that hamstring injuries requiring diagnostic investigation were significantly more severe (26 days lost) versus injuries diagnosed by clinical examination alone (13 days lost). This study also reported that recurrent injuries resulted in average of 25 days lost from play compared to 14 days lost for new injuries.[21]

Askling et al. recently published two studies that utilized MRI to characterize hamstring injuries. A study of 18 elite sprinters who sustained acute hamstring injuries required an average of 16 weeks to return to pre-injury activity level, with a range of 5-50 weeks. Proximal free tendon involvement and proximity to ischial tuberosity were associated with a longer rehabilitation time.[45] A subset of subjects who had injuries at proximal myotendinous junction had a mean return to pre-injury level of activity of 14.2 weeks, ranging from 6 to 26 weeks. Another study by the same author evaluated 30 subjects from 21 different sports and found that a majority of athletes (88%) still had symptoms after they returned to their sports and time to return was longer for recreational subjects than for elite level subjects (median 62 weeks, ranges 40-104 weeks vs. 25 weeks, 9-80 weeks). In this study free tendon involvement was a possible risk factor for a prolonged time to return to pre-injury level.[16]

Extrapolating a time to return to play for hamstring injuries from the previous studies is a difficult and inexact task as evidenced by the many variables involved and a wide range of time reported in the studies ranging from mean of 14 days to 62 weeks. Data on lost time from competition for recurrent hamstring injuries is even more limited and two groups have noted that recurrent injuries prolonged rehabilitation duration by 33% (Powell and Zarins, 1989-1998, 1696 injuries in NFL players as reported at 2002 NFLTP meeting) to 79% [21] compared to new injuries. However, studies that reported longer rehabilitation time for recurrent injuries compared to new injuries did not stratify the severity or location of the injury, making analysis challenging.

We described a case of an intercollegiate football punter that sustained a recurrent grade II hamstring injury while kicking. MRI revealed a partial tear at the proximal

biceps femoris myotendinous junction. The injury occurred at the end of season without pressure to return quickly to competition, which would most likely contribute to longer rehabilitation time. Therefore while in this case return to play time was not an issue, based on measurements and clinical symptoms the subject would likely have been cleared at less than 9 weeks to return to play. If the injury had not been a recurrence, presence of poor prognostic factors in his clinical and radiographical presentation would have predicted lost time from play for 6 weeks or greater. As a recurrent injury, the conservative estimate of rehabilitation time required would have ranged from 8 to 11 weeks.

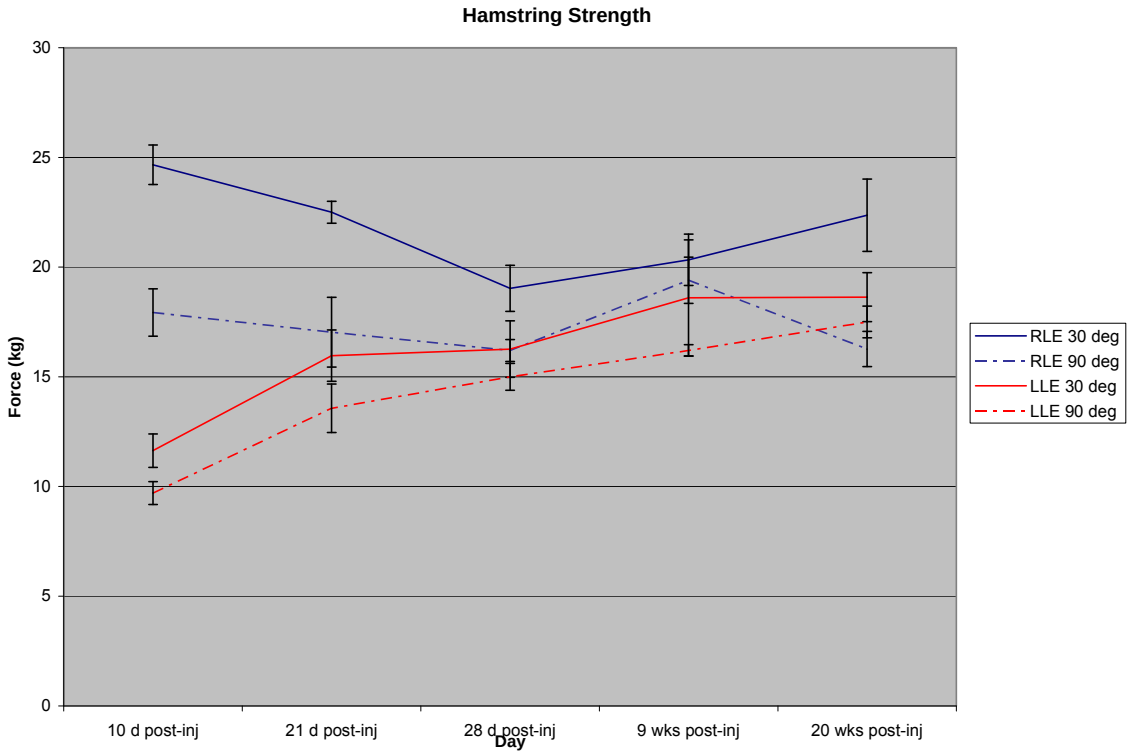
Conclusion

We have described use of losartan, which is an FDA-approved angiotensin II receptor blocker, to treat a healthy inter-collegiate football player with a grade 2 (partial tear) biceps femoris injury. The patient tolerated the course of losartan well with no hypotension or any other side effects. Additionally, the patient demonstrated recovery of normal flexibility and strength compared to the contra-lateral leg. A repeat MRI revealed a healing proximal hamstring myotendinous junction strain with minimal residual edema. The subject would have been cleared to return to full participation in off-season training for inter-collegiate football at less than 9 weeks after injury based on the hamstring flexibility, strength measurements and activity-limiting pain, and to date (> 20 weeks) has not suffered any further injury to the hamstrings. The recovery for this athlete was at the shorter range of time reported in the literature for a subset of patients in a similar age group with a similar mechanism and severity of injury. We are encouraged by the results of this case

report, however we recognize the limitations of making treatment decisions on the basis of a single case report. We believe that translational clinical research, in the form of a prospective, blinded, placebo controlled randomized clinical trial are necessary to determine the benefits of losartan for the treatment of hamstring muscle injuries. This research should consider the effects of the angiotension II receptor blocker on recovery of hamstring flexibility and strength, degree of fibrosis, time to return to full participation in sports and the frequency of recurrence as well as any adverse effects encountered by otherwise healthy individuals.

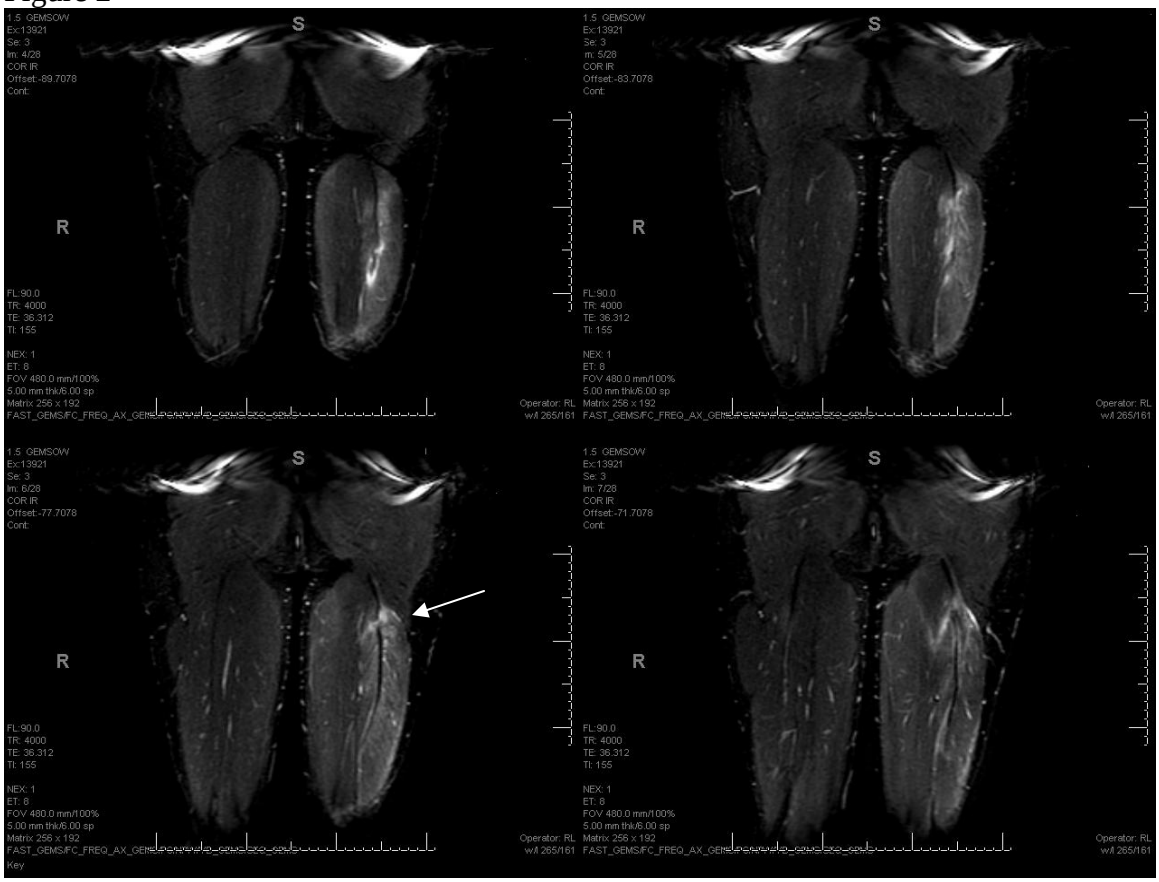
Figures and graphs

Figure 1



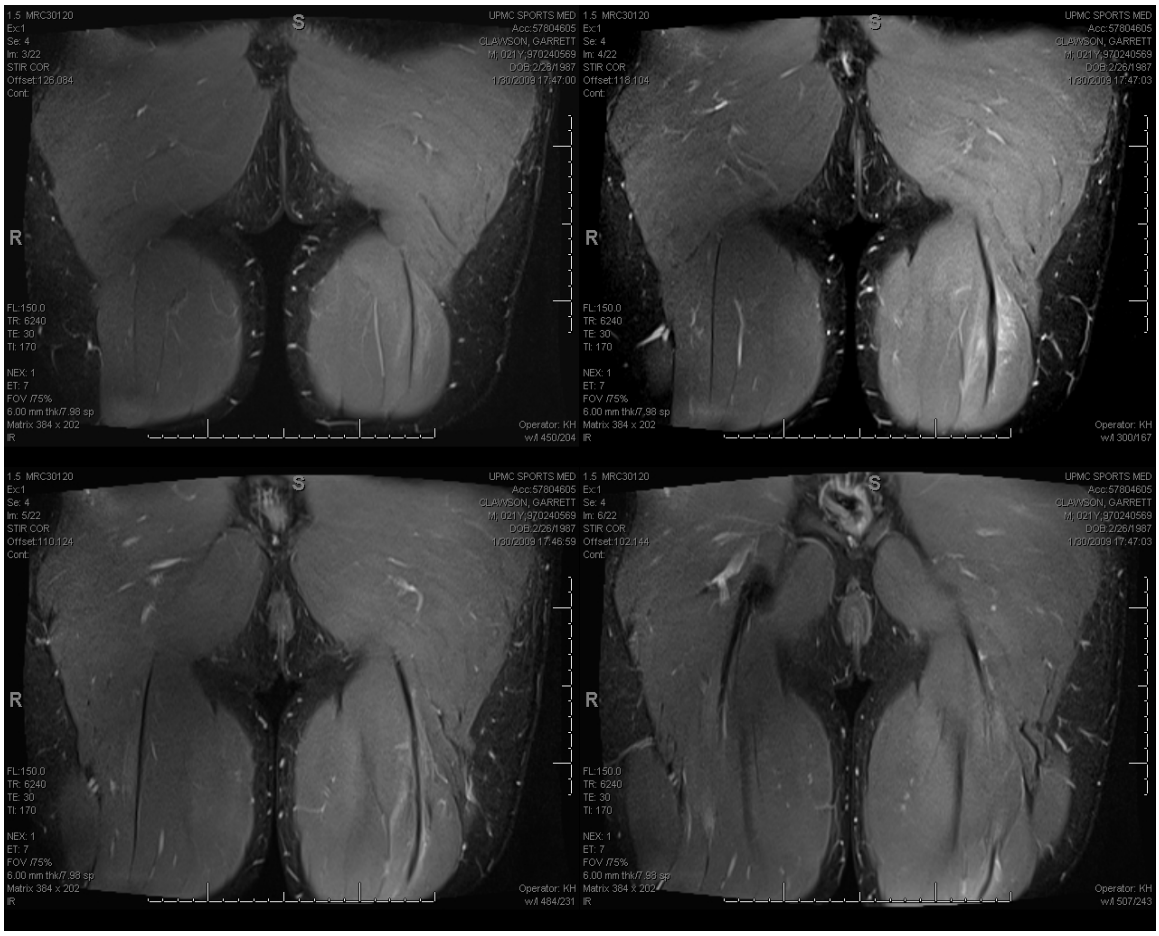
Hamstring strength was measured with a hand-held dynamometer. LLE is the injured side and RLE is the uninjured side. While we see a steady improvement in strength on the injured side over time, we also note that there is some decline in the strength of the uninjured side during the initial period after the injury when the rehabilitation treatments were focused on the injured side only.

Figure 2



1st baseline MRI
MRI shows a partial tear at the left proximal myotendinous junction of the long head of the biceps femoris; also present is the surrounding edema with hyperintense signal in the hamstring muscle belly.

Figure 3



Follow-up MRI at 11 weeks shows a thickened proximal myotendinous junction of the biceps femoris where the previous tear was present with decreased edema in the surrounding muscle compared to the first MRI.

Table 1

Author, year	# of subjects	Sports	Acute vs Recurrent	Mean time to return to play	Range of time to return to play	Imaging type	Associated factors to longer rehab
Pomeranz 1993	14	Professional athletes (sports unspecified)	Unspecified		1-15 weeks	MRI	Complete tendinous or myotendinous jxn rupture, hyperintensity or hemorrhagelike signal intensity on T1-weighted images, large cross-sectional area involvement (>50%), distal tendinuos or myotendinous lesions, and localized ganglionlike peritendinous fluid collections
Slavotinek 2002	37	3 Australian rules football	Acute	27 days	13-48 days	MR	Abn cross-sectional muscle area greater than 50%
Connell 2004	60	Australian football	53% of 60 w/previous injury	21 days	4-56 days	MRI + U/S	BF injury, cross-sectional area, length of injury on MRI, injury outside of MTJ
Brooks 2006	546	UK pro rugby union	Both	New (14 days) vs recurrent (25 days); kicking (36 days)		26% imaged (53% U/S, 40% MRI, 7% both); 74% clinical exam alone	Kicking mechanism
Askling 2007	18	Elite sprinters	Acute	16 weeks	(5-50 weeks)	MRI	Proximal free tendon and proximity to ischial tuberosity involvement
Askling 2008	30	21 different		Elite	Elite (9-80	MRI	Free tendon

		sports		athletes 25 weeks; recreational athletes 62 weeks	weeks); Recreational (40-104 weeks)		involvement, recreational-level athletes

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